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A study of anti-tissue antibodies occurring naturally and after tissue damage.

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To my wife, Joanna,

and my parents

CONTENTS

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INTRODUCTION

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Ehrlich & Morgenroth defined, in their classical communications at the turn of the century, one of the central problems in immunology, when they tested the ability of an animal to react immunologically against its own body material. They had established (Ehrlich, collected papers 1957) that the serum of a goat injected with sheep erythrocytes developed the capacity to lyse the sheep erythrocytes and, by that time, it was recognised that animals could react similarly to a wide range of foreign material. In their third communication on haemolysins they state "In pathology the changes foremost to be considered are those resulting from the absorption, by an organism, of its own cell material". Thus in an individual with a considerable subcutaneous haemorrhage "the essential conditions, just as in experiment, are given for the formation by reaction of substances possessing specific injurious affinities for these blood cells". Moreover "the same can apply to other tissues; for every acute atrophy of the parenchyma of an organ can lead to the absorption of cell material and to the consequences". It seemed to them of the "highest pathological importance to determine whether the absorption of its own body material can excite reactive changes in the organism and what the nature of these changes is". Accordingly they tested the serum of a goat, injected with a pool of homologous erythrocytes, for haemolytic activity. They found that this serum was able to lyse erythrocytes from eight of nine goats tested and yet the serum had no lytic effect, in vitro,

on autologous erythrocytes. From this they concluded that the serum contained antibodies which would react only with antigens limited to the heterologous erythrocyte surface. They supposed that the animal possessed "certain regulating contrivances" which prevented an injurious immune reaction against the animal's own cell material. This led them to postulate "a horror autotoxicus of the organism", which concept was to become almost a fundamental axiom of immunology for the next half century.

Various observations accumulated over the years which were pertinent to the problem of the discrimination of self material by the immunological system. Murphy (1913) reported that chicken embryos were unable to react immunologically to an inoculation of Jensen rat sarcoma. The graft became heavily vascularised and grew rapidly. In contrast adult animals rejected a similar transplant within three days. Grasset (1929) injected chicken embryos with diphtheria toxoid and challenged them with toxoid later in life. These animals were found to have no higher titres of anti-toxoid than untreated control animals challenged contemporareously. A striking incidence of this phenomenon of "tolerance of immature tissues to foreign material" (Burnet, 1941) was Owen's (1945) demonstration of erythrocyte mosaicism in dizygotic twin cattle. An interchange of erythrocytes between the twins occurred as a result of placental vascular anastomoses and this chimaerism was found to be continued in adult life. The significance of these findings was realised

in 1949 when Burnet & Fenner attempted to explain the mechanism by which the immunological system distinguishes between material to which it is reactive and material to which it is unreactive on the basis of "self markers". They proposed that at a certain stage of development the immunological system recognised self marked material which then became immunologically inert. As a direct consequence of this theory they predicted that an adult animal might be immunologically tolerant to a foreign antigen injected at birth. Moreover they noted that "something more than mere casual presence of an antigen in the embryo is necessary if subsequent immunological reactivity is to be modified in the way we have suggested". Experimental evidence apparently confirming Burnet & Fenner's prediction was soon forthcoming. Billingham, Brent & Medawar (1953) showed that adult mice and chickens were rendered specifically tolerant to isoantigenic tissue to which they had been exposed in foetal life. They found that animals which had been injected with tissue preparations in embryo were unable to reject skin grafts from the donors of the original inoculum. Tolerance to heterologous protein antigen was demonstrated by Hanan & Oyama (1954). Rabbits were given repeated injections of bovine serum albumin (BSA) from the time of birth and were challenged with alum precipitated BSA. In contrast to littermate controls these animals did not form anti BSA antibodies as judged by precipitation, sensitisation of guinea pigs for anaphylaxis and the Arthus reaction. Burnet (Burnet, Stone and

Edney, 1950) tested his prediction by administering human erythrocytes to chicken embryos and observing the effect of this treatment on the subsequent production of natural haemagglutinins to human erythrocytes. Paradoxically, these chickens developed natural haemagglutinins to human erythrocytes before untreated controls.

Burnet (1959, 1962) discarded his "semi-mystical self markers" in the clonal selection hypothesis. According to this theory there exists in the body a large number of clones of genetically different, potential antibody forming cells, each clone capable of producing antibodies of only a limited number of specificities. Cells of a particular clone respond to contact with the appropriate antigen by proliferation and production of their characteristic antibody. To account for the acquisition of immunological tolerance to self material Burnet postulated that those clones of cells responsive to self material were eliminated or suppressed. He concluded that animals would not produce antibody or show any other immunological reaction against antigenic patterns present in its cells or body, provided that these were not functionally insulated (i.e. "hidden") from the general turnover of cells in the body.

As this thesis is concerned with immunological reactions against self material it seems pertinent to examine the literature pertaining to the mechanism underlying tolerance with a view to establishing whether an animal must necessarily become tolerant to all antigens to which it is exposed in perinatal life. Secondly,

it is proposed to survey the reports of immunological reactions against self material and the effects which have been ascribed to such reactions in pathological and normal conditions. Finally, the investigations which led to the initiation of this work will be reviewed.

Immunological Tolerance

The state of specific immunological suppression induced by exposure to a particular antigen has been termed "paralysis", "tolerance" and "unresponsiveness". The term "paralysis" was coined to describe the phenomenon of immunological non-reactivity induced by the administration of large amounts of pneumococcal polysaccharide to adult animals (Felton and Bailey 1926, Felton and Ottinger, 1942). "Tolerance" was originally used in connection with the failure of an animal to reject a homograft (see Burnet, 1941) and was later used to denote the phenomenon of specific immunological suppression induced by limited exposure to an antigen in perinatal life (Billingham, Brent and Medawar, 1953). "Unresponsiveness" was applied to the specific repression of the immune response induced by exposure to non-living antigens (Smith, 1961). These terms are often used synonymously despite the possibility that the mechanisms through which these phenomena are induced may be different. Lymphoid cells from animals rendered tolerant to skin grafts (Argyris, 1963) and unresponsive to Shigella antigens and bovine serum albumin (Friedmann, 1962 and

Dietrich & Weigle, 1964) have been found to be unable to respond to the appropriate antigenic stimulation when transferred to X-irradiated syngeneic recipients. This suggests that tolerance and unresponsiveness are basically similar phenomena. Their relationship to paralysis is not so clearly defined. Sercarz and Coons (1959) using the immunofluorescent method failed to detect antibody forming cells in the spleens of mice 70 days after a paralysing dose of pneumococcal polysaccharide. Brooke and Karnovsky (1961) found that lymphoid cells from paralysed mice, in contrast to those of immunised mice, were unable when transferred to allogeneic recipients to protect them from a lethal dose of pneumococci. However, these experiments can be criticised on the grounds that any antibody formed would be masked by persisting antigen. Moreover, Howard (personal communication) has recently detected anti-pneumococcal polysaccharide antibody forming cells in the spleens of mice, paralysed with pneumococcal polysaccharide, using the immunocyto-adherence technique of Biozzi (Biozzi et al, 1966).

Factors Influencing the Induction of Tolerance

There are a number of factors which appear to affect the facility with which an animal can be made tolerant to a particular antigen. Of these, the maturity of an animal seems to be important. It is now well established that an animal can be rendered specifically tolerant to a wide range of antigens by exposing the animal

to these antigens in perinatal life (Smith, 1961; Leskowitz, 1967). The finding that unresponsiveness can be induced in adult animals, e.g. by deaggregated serum proteins (Dresser 1962, Thorbecke & Benacerraf, 1967) led Good & Papermaster (1964) to suggest that the susceptibility of an animal to the induction of tolerance was inversely related to the maturity of the lymphoid tissue. Another possibility, namely that the immunological mechanism may mature to different antigens at different times, was raised by Hasek, Lengerova & Hraba (1961). This suggestion was based on the work of Porter (1960) who found that tolerance in rabbits to homologous cells could not be induced after the 22nd day of gestation as judged by homograft rejection in adult life (normal gestation period in rabbits 31 days). In contrast Smith & Bridges (1958) found that tolerance to bovine serum albumin in rabbits could be induced up to 15 days after birth. These findings could also be explained by the mechanisms of delayed type hypersensitivity and of antibody formation maturing independently in ontogenesis. However, Turk & Humphrey (1961) were unable to dissociate delayed type hypersensitivity and antibody production to bovine serum albumin in guinea pigs by neonatal exposure to the antigen.

There is some evidence that the induction of tolerance by perinatal contact with an antigen may be hindered by transfer of maternal antibody to the foetus. Mitchison (1962) considered that the failure of some chickens to become tolerant to turkey

erythrocytes was due to passive transfer of anti-turkey haemagglutinins. He found a rapid increase in the rate of elimination of turkey erythrocytes from the circulation of embryonic chickens and noted that this was coincident with the passage of passively transferred antibody from the yolk to the circulation. However, this rate increase was also coincident with the appearance of natural haemagglutinins to turkey erythrocytes in the serum. Indirect evidence comes from the work of Havas and Senff (1967) who observed that mice which had been injected with human gamma globulin (HGG) and anti-HGG serum neonatally, had considerably higher titres of anti-HGG antibody, when challenged in adult life, than littermate controls injected neonatally with the antigen alone. Similarly, Friedman (1965) observed that the injection of specific antiserum with a soluble Shigella antigen interfered with the induction of tolerance to this antigen.

It has been stated that tolerance is most effectively induced by antigens with which the host shares the maximum number of antigenic determinants (Smith, 1961; Humphrey & White, 1964; Leskowitz, 1967). Thus Mitchison (1962) was able to induce more complete tolerance to irradiated homologous erythrocytes than to irradiated turkey erythrocytes in chickens. Dresser and Gowland (1964) found that injection of leporine γ-globulin in rabbits elicited unresponsiveness whereas injection of the more distantly related (phylogenetically) antigen, equine γ-globulin, stimulated an immune response. Similar studies in mice have shown that tolerance is

more easily induced with human γ-globulin than with turkey γ-globulin (Dietrich & Weigle, 1964). Tempelis (1965) showed that the molar combining ratios of chicken antibody with turkey γ-globulin (2:1), goose γ-globulin (3:1), human γ-globulin (5:1) and bovine serum albumin (9:1) correlated with the ability of these antigens to elicit a tolerant state in chickens. Gery, Davies & Lazerov (1964) found that rats injected with bovine heart homogenate at birth, did not produce complement fixing antibodies on later challenge with bovine heart, although high levels of antibodies were detectable by passive haemagglutination and gel diffusion. Similar neonatal treatment of rats with guinea pig and rabbit heart homogenate produced a greater degree of tolerance using the same criteria. There are, furthermore, a number of studies which record the difficulty of inducing tolerance to distantly related antigens. Dietrich & Weigle (1963) induced complete tolerance to the soluble protein antigen, haemocyanin, in only 20 per cent of mice injected neonatally with a single dose (16mg.) of the antigen. Smith & Bridges (1958) failed to induce tolerance in rabbits by a single neonatal injection of a number of different bacterial antigens and similar failures have been reported in chickens (Cohn, 1957). Finally, Gowland, Hobbs & Byers (1965) listed a number of reports of failures to induce tolerance to particulate bacterial and viral antigens. However Friedmann & Gaby (1960) succeeded in inducing partial tolerance to a Shigella paradysenteriae suspension in mice by a single

injection of a soluble Shigella antigen at birth. Moreover, complete tolerance to the soluble flagellin antigen of Salmonella adelaide has been induced in rats (Ada, Nossal & Austin, 1965) and Shellam & Nossal (1968) demonstrated that tolerance could be induced by neonatal injection of minute quantities of the same flagellin antigen. The possibility arises that some factor, other than the relationship of the antigen to host material, may be involved in success or failure of induction of tolerance in the experiments described above. Thus it may be significant that in these experiments the failure to induce tolerance is associated with the use of particulate antigens whereas the successful induction of tolerance is linked with the use of soluble antigens.

Fishman and his co-workers (Fishman, van Rood & Adler, 1965; Adler, Fishman & Dray, 1966) have presented evidence that the phagocytosis of an antigen is an essential step in the initiation of the primary immune response. Although the exact mechanism of this process is still in doubt (Askonas & Rhodes, 1965; Harris & Cramp, 1968) it seems likely that the induction of an antibody response involves the sequential action of macrophages and lymphocytes (Shands 1967). As a corollary to this hypothesis it has been proposed that the induction of tolerance is facilitated by the failure of macrophages to phagocytose and degrade an antigen thus allowing the antigen to reach the lymphocytes intact (Frei, Benacceraf & Thorbecke, 1965). This proposition was based on the finding that adult animals could readily be rendered tolerant

by the injection of heterologous serum proteins from which the supposedly "phagocytosable" material had been removed (Frei, Benacceraf & Thorbecke, 1965; Dresser 1962). There is some evidence supporting this theory. Mitchell & Nossal (1966) studied the localisation of antigens in new born rats. They found that the soluble antigen, flagellin, was widely distributed throughout the lymphoid tissues and that there was little evidence of its phagocytosis. This contrasted with the extensive phagocytosis found in adult rats. They considered that this inefficient phagocytosis, prolonging exposure of the lymphocytes to intact antigen, might be important in inducing tolerance in the neonatal animal. Their contention was weakened, however, by the finding that the injection of anti-flagellin serum with flagellin did not alter its distribution in neonatal rats. It would have been strengthened considerably had they demonstrated that a similar non-tolerance inducing antigen e.g. flagella had a different distribution. Claman (1963) has shown that endotoxin, a potent stimulator of the reticulo-endothelial system (RES), injected simultaneously with deaggregated bovine γ-globulin in adult mice prevented the induction of tolerance. Unfortunately, this effect of endotoxin has been found to be independent of the granulopectic activity of the RES (Golub & Weigle, 1967). The injection of bovine serum albumen (BSA) from one to fifteen days after whole body irradiation of adult rabbits with 500 rads, resulted in specific unresponsiveness to BSA, (Linscott & Weigle,

1964). The work of Gallily & Feldman (1967), showed that such impaired immunological reactivity following X-irradiation was due to macrophage damage rather than damage to lymphoid cells. Finally, Janeway & Sela (1967) observed that the injection of high doses of a simple polypeptide composed of D amino-acids induced paralysis in mice whereas the corresponding L polymer elicited an immune response. The D polymer was shown to be retained about 500 times more efficiently by the cells of the reticulo-endothelial system and metabolised 20 times slower than the L polymer (Janeway & Humphrey, 1968). This suggests that a dominant factor in the induction of paralysis may be slow catabolism of antigen coupled with high antigen retention, which might then facilitate the induction of paralysis by allowing free intact antigen to come into direct contact with lymphocytes.

Clearly then, there is a considerable body of circumstantial evidence which favours the hypothesis that the induction of tolerance is facilitated by antigen being allowed direct access to lymphocytes. However, to date, an unequivocal demonstration that tolerance results from direct action of antigen on lymphocytes, is lacking.

Thus, the findings reviewed above direct attention to a number of factors which appear to affect the induction of tolerance to a particular antigen. It is apparent that an animal does not become tolerant to all antigens to which it is exposed in perinatal life. It seems necessary to inquire how

and why this should be.

Role of Immune reactions in Autoimmune disease

Since the report by Donath & Landsteiner (1904) of a haemolysin, which absorbed to erythrocytes at low temperature, in the serum of patients with paroxysmal cold haemoglobinuria there has been speculation as to the role of immunological factors in the pathogenesis of the so-called autoimmune diseases. The evidence for autoimmune participation in these disorders rests largely on the demonstration of antibodies or delayed hypersensitivity to tissue constituents, the damaging effect of their inter-reaction and inflammatory changes in the affected tissue. Other features such as raised immunoglobulin levels, lymphoid hyperplasia, reduced serum complement level and the response to immunosuppressive agents have been quoted as supporting the postulated autoimmune pathogenesis of these disorders (see Glynn & Holborow, 1965 and Anderson, Buchanan & Gouidie, 1967).

It would be expected that autoantibodies or sensitised cells would have a destructive effect on normal healthy cells if they are involved in the induction of tissue damage. Many in vitro studies of the action of appropriate anti-sera and sensitised cells on the corresponding target cells in tissue culture have been made. Irvine (1962) reported a cytotoxic antibody in patients with thyroiditis. Aged white cells from children with

ulcerative colitis have been shown to specifically damage foetal colon cells in tissue culture (Broberger & Perlmann, 1963). Similar cytotoxic effects of lymphocytes have been reported in experimental thyroiditis in monkeys (Kite, Argue & Rose, 1966) and in experimental nephritis in rats (Holm, 1966). Significantly, these interactions take place only if the cells in tissue culture are trypsinised prior to testing (Pulvertaft et al, 1959; Ling et al, 1965). Moreover, it is interesting to note that normal autologous serum has been found to have a deleterious effect on trypsinised human synovial cells (Clarris & Fraser, 1968). Appel and Bornstein (1964) described an antibody in the serum of two rats with allergic encephalomyelitis which caused demyelination of myelinated axons and swelling of neuroglial cells without affecting the neurones in cultures of neonatal rat cerebellum. Presumably, in vivo formation of such antibody would only be stimulated if the blood brain barrier was damaged in some way and only if breakdown of the barrier persisted would this antibody be able to interact with brain tissue. In this respect it seems pertinent to mention the finding of a naturally occurring cytolytic autoantibody for testicular cells in normal guinea pigs, rabbits, rats, cats and dogs (Spooner, 1964, 1965). Such autoantibody was shown to exert a cytolytic effect on isolated testicular cells in vitro although in vivo binding of such antibody could not be demonstrated. Weir (1962) examined kidney and spleen sections from patients with high titres of anti-nuclear

factor using a fluorescein anti-human globulin conjugate. The sections showed no evidence that in vivo uptake of globulin had taken place although the patients own antibody was readily taken up on such sections after treatment in vitro. In addition tissue culture preparations showed no uptake of added anti-nuclear antibody until the cells had been killed and trypsinised.

It has been stated that anti-erythrocyte autoantibodies in patients with acquired haemolytic anaemia are probably "the sole initiating cause" of increased erythrocyte destruction (Dacie, 1962). These autoantibodies have been generally classified on the basis of laboratory observations into "cold" and "warm" types and the patients in whom they have been found fall correspondingly into two clinical syndromes. The cold type of autoantibodies, which are probably IgM (Worlledge, 1965; Schubothe, 1966), have been found to agglutinate and, in the presence of complement, lyse erythrocytes. However, such reactions were found to take place with "normal" erythrocytes only at temperatures below 32°C. and at a pH of 6.8 (Dacie, 1950). Jancovii (1954) has found low titres of similar cold agglutinins in most normal human sera.

The warm type of autoantibody is predominantly IgG (Dyster, Nachmann, Christenson & Engle, 1966) and has been found to react with normal erythrocytes under physiological conditions (Dacie, 1962). These autoantibodies do not agglutinate or lyse erythrocytes and are only detectable by primary binding techniques, e.g.

Coombs' antiglobulin test. The relationship between the presence of warm type autoantibody and erythrocyte survival *in vivo* is not yet clear. Thus it has been reported that autoantibody coated erythrocytes (i.e. Coombs positive) survive normally in normal human recipients whereas normal erythrocytes are eliminated as rapidly, and in some cases more rapidly, than autologous erythrocytes in patients with acquired haemolytic anaemia (Mollison & Patterson, 1949; Culp & Chaplin, 1960). Cases have been recorded of Coombs positive erythrocytes from patients with "warm" type autoimmune haemolytic anaemia being eliminated relatively rapidly in normal human recipients (Dacie, 1962; Schwartz & Costea, 1966). However, it has occasionally (up to 25 per cent) been found that erythrocytes from healthy human donors have relatively short survival times in apparently compatible recipients (Mollison, 1959). A similar heat labile IgG warm type autoantibody has been found in low titres in 99 per cent of normal human beings (Delage, 1958).

Attempts to set up comparable experimental models were unsuccessful until the discovery that the inbred New Zealand black (NZB) strain of mice spontaneously develop autoimmune haemolytic anaemia and Coombs positive erythrocytes between four and nine months after birth (see Burnet, 1963). Holmes, Gorrie & Burnet (1961) transferred spleen cells from old Coombs positive mice with haemolytic anaemia to young mice. The recipient mice showed no sign of anaemia but their erythrocytes became Coombs positive.

Similarly Lindsey, Donaldson & Woodruff (1966) found that the survival of erythrocytes transfused to NZB recipients from isogeneic donors was similar to that of the recipients own erythrocytes, irrespective of whether or not the donor's erythrocytes were Coombs positive. These results were confirmed by Barnes & Tuffrey (1966) who transferred spleen cells from old Coombs positive NZB mice to young recipients and found that the acquisition of Coombs positivity did not result in increased elimination of the recipients erythrocytes. However, they showed that transfer of spleen cells from old mice with anti-human red cell cross reacting (AHR) autoantibody to young recipients seemed to be associated with an increase in erythrocyte elimination. Whether the AHR autoantibody or the transferred cells were responsible for this increase in erythrocyte elimination was not elucidated. It may be significant that Warner & Wister (1968) have found that, although the majority of bound autoantibody on erythrocytes of old NZB mice is γG, analogous to the warm type of autoantibody in humans, some γM antibody can also be detected.

The production of inflammatory lesions in experimental animals following the injection of tissue antigens provides evidence for the participation of autoimmune reactions in tissue damage. Historically it was realised that immune reactions might be implicated in the neurological catastrophes that followed the introduction of rabbit nervous tissue in Pasteur's rabies

vaccine and this view was supported by Metalnikoff's (1900) report of a spermotoxine in guinea pigs injected with autologous spermatozoa. Shwentker & Rivers (1934) induced encephalomyelitis in rabbits by repeatedly injecting them with autolysed homologous brain suspensions and encephalomyelitis was produced in monkeys by the injection of homologous brain emulsified in Freunds complete adjuvant (Kabat, Wolf & Bezer, 1947; Morgan, 1947). Similarly, thyroid lesions were found in rabbits following the injection of saline extracts of both autologous and homologous thyroid in Freunds complete adjuvant (Rose & Witebsky, 1956). These demonstrations stimulated numerous investigators to study the effect of injecting various tissue antigens with and without adjuvant, into experimental animals (see review by Dumonde 1966). Inflammatory lesions in various organs have been found occasionally in "normal" animals and the injection of Freunds adjuvant has been shown to increase the occurrence of such lesions (Laufer, Rosenmann & Davies, 1966). The effect of injecting an organ specific antigen incorporated in Freunds adjuvant is to increase the incidence and severity of lesions in the corresponding organ. Another fact which emerges from such studies is the lack of correlation between the severity of the lesion and the amount of circulating autoantibody e.g. anti-thyroid autoantibody and thyroid lesions in rabbits, guinea pigs and dogs (Terplan et al, 1960), anti-kidney autoantibody and nephrotic syndrome in rats (Hunter, Hackel & Heymann, 1960) complement fixing anti-adrenal autoantibody and

adrenal lesions in guinea pigs and rabbits (Terplan, Witebsky & Milgrom, 1963), anti-heart autoantibody and myocardial lesions in rabbits, rats and hamsters (Davies, Laufer, Gery & Rosenmann, 1964) and complement fixing anti-kidney autoantibody and kidney lesions in rats (Boss, Silber & Nelkon, 1967).

A number of attempts have been made to correlate the onset and severity of lesions with hypersensitivity. Waksman & Morrison (1951) found that the production of encephalomyelitis in rabbits by the injection of homologous brain in Freunds complete adjuvant was associated with the development of delayed type hypersensitivity skin reactions. This finding was corroborrated by Shaw, Alvord, Kahu & Kies (1965) who described a parallelism between the onset of encephalomyelitis in guinea pigs injected with a purified basic protein from homologous brain incorporated in Freunds complete adjuvant, and the intensity of delayed type skin reactions. Similar studies were carried out on the pathogenesis of experimental thyroiditis in guinea pigs injected with picrylated thyroglobulin (Meischer, Gorstein & Benacerraf, 1961). This procedure led to the development of thyroiditis and delayed type hypersensitivity to thyroglobulin with minimal production of circulating antibody. Moreover, there was a positive correlation between the intensity of delayed skin reactivity to intradermal thyroglobulin and the severity of thyroiditis. Conversely the production of anti-thyroglobulin autoantibodies without thyroiditis has been observed in rabbits injected with alum precipitated

thyroglobulin (Rose, Kite & Deobbler, 1962). It is important to note that in these experiments, the association between delayed hypersensitivity to tissue antigens and the presence of tissue damage is not complete. In this respect it is interesting that guinea pigs injected with bovine thyroglobulin were found to exhibit delayed hypersensitivity to bovine thyroglobulin but not to guinea pig thyroglobulin and yet they had mild thyroiditis (Flax, Jankovic & Sell, 1963). Moreover, it seems significant that in guinea pigs with experimental allergic orchitis, testicular lesions have only been found in animals exhibiting delayed hypersensitivity, and producing circulating antibody, against testicular antigen (Brown, Glynn & Holborow, 1967).

The transfer of experimental autoallergic lesions from a donor to a recipient with sensitised lymphoid cells emphasises the role of delayed type hypersensitivity in the production of inflammatory lesions. The experiment of Lipton & Freund (1953) showed that encephalomyelitis in rats could be transferred by parabiosis but did not indicate whether the disease was transferred by serum or sensitised cells. Paterson (1960) induced allergic encephalomyelitis in rats by the intravenous injection of a suspension of lymph node cells from donor rats injected previously with heterologous brain in Freunds complete adjuvant. Six out of 27 recipients developed allergic encephalomyelitis. Parallel experiments using large volumes of serum from immunized donors, failed to produce encephalomyelitis in the recipients.

Subsequent studies in inbred guinea pigs (Stone, 1961) rabbits (Åström & Waksman, 1962) and inbred mice (Lee, Scheinberg, Schnieder & Edelman, 1965) have confirmed that allergic encephalomyelitis can be transferred to normal recipients by means of lymph node cells from appropriately sensitised donors. The low frequency of success in transferring allergic encephalomyelitis in rats, rabbits and mice (see Lee et al, 1965) suggests that these animals are able to resist the development of allergic encephalomyelitis. Thus it seems significant that splenectomy in recipient rats (Koprowski, Jervis & Schoeniger, 1961; Paterson, 1966) and X-irradiation in recipient rabbits (Åström & Waksman, 1962) has been found to facilitate transfer of the disease. Furthermore, a higher frequency of transfer has been accomplished by injecting the donor cells directly into the central nervous system of the recipient (Åström & Waksman, 1962; Paterson & Weiss, 1965). In addition Levine & Wenk (1967) have found that, in recipient rats pretreated with hydrogen cyanide, transferred sensitised cells localize in areas of the brain already damaged. This indicates that the target antigen recognised by the transferred cells is damaged tissue. Few reports have appeared in the literature on the transfer of other experimental auto-allergic lesions. A preliminary report on the transfer of experimental thyroiditis via lymphoid cells from donor guinea pigs with thyroiditis to inbred recipients (Felix, Davies & Waksman, 1961) has not been confirmed. The transfer of autoimmune nephrosis in

the rat by means of lymph node cells has been reported (Hess, Ashworth & Ziff, 1962; Heymann, Hunter, Hackel & Cuppage, 1962) but the validity of these findings has been questioned by Unanue & Dixon (1967).

Further support for the role of delayed type hypersensitivity in autoimmune disease comes from the observation that there is a frequent occurrence of autoimmune disease in aggammaglobulinaemic patients and the implications of this have been discussed by Fudenberg (1966).

Evidence that "autoreagins" occur and are involved in autoimmune disease is fragmentary. A high incidence of penicillin hypersensitivity has been found in patients with thyroid disease (Blizzard, Hamwi, Shillman & Wheeler, 1959) and Kaiser & Beall 1964 (quoted by Serafini, 1965) described an association between Hashimoto's thyroiditis and typical allergic diseases in one family. Serafini, Torrigiani and Masala, 1965, noted a high incidence of thyroid autoantibodies in allergic patients.

Thus the findings reviewed above direct attention to the role of delayed type hypersensitivity in autoimmune disease whilst not excluding a supplementary effect of some types of autoantibody. They show that humoral and cellular immune reactions against tissue antigens do occur in autoimmune disease. However, a direct aggressive immune reaction against healthy and normal (in vivo) cells has not yet unequivocally been demonstrated.

"Protective" effect of autoantibody

The belief that all autoantibodies were pathogenic was shaken by the work of Thomas, Paterson & Smethwick (1950). They provoked the formation of a complement fixing antibody reactive with alcoholic extracts of homologous brain in dogs by the injection of homologous brain in Freunds complete adjuvant. Of these dogs, 35 out of 55 developed allergic encephalomyelitis. Unexpectedly, they found considerably lower titres of this antibody in paralysed animals than in unparalysed animals. This observation led Thomas (1964) to suggest that the antibody in question actually had some protective effect. Experimental support for this view was soon forthcoming. Paterson & Harwin (1963) obtained pools of serum containing high levels of complement fixing anti-brain auto-antibody from Wistar rats injected with guinea pig brain in Freund's complete adjuvant. The serum was injected intravenously into recipient rats actively sensitised to nervous tissue. Repeated injection of the serum was found to exert a profound suppressive effect on the development of allergic encephalomyelitis. The suppressive activity of such serum was shown to be associated with the activity of a labile complement fixing IgM anti-brain antibody (Paterson, Coia & Jacobs, 1965; Paterson, 1966). Further evidence for the protective effect of the complement fixing antibody was provided by the observation that the susceptibility of different strains of rats to the induction of allergic encephalomyelitis varied inversely with the ability to produce such antibody (Paterson,

Coia & Jacobs, 1965b; Paterson, 1966).

A similar protective phenomenon, attributable to the action of autoantibodies has been reported with respect to the limitation of myocardial damage in experimental animals. Thus Davies et al (1964) induced myocardial damage in rats and rabbits by the injection of homologous heart in Freunds complete adjuvant. They noted that there appeared to be a negative correlation between the level of circulating anti-heart autoantibody, as judged by passive haemagglutination, and the severity of myocardial lesions.

An apparently unconnected stabilising effect, of autoantibody on lysosomes, was reported by Miescher, Wiedermann, Hirchhorn & Weismann (1964). They showed that an anti-lysosomal autoantibody, from the serum of patients with hepatitis, prevented the Vitamin A induced release of B glucuronidase from isolated lysosomes. Moreover the phagocytosis of autoantibody coated lysosomes was found not to affect rabbit polymorphonuclear leucocytes (PMN). In contrast, on phagocytosis of uncoated lysosomes PMN were found to undergo cell damage.

Another finding which could be construed as indicative of a protective effect of autoantibody was that observed by Lindsey, Donaldson & Woodruff (1966). They measured the survival rate of erythrocytes transfused to old Coombs positive NZB mice with haemolytic anaemia. In such mice they found that Coombs positive erythrocytes survived significantly longer than Coombs negative erythrocytes.

Thus it seems that some types of autoantibody appear to potentiate tissue damage whilst others appear to have an inhibitive effect. This is, perhaps, exemplified by the work of Hedberg & Källen (1964). They found that mononuclear cells from the synovial fluid of some arthritic patients had a cytotoxic effect on cultures of human fibroblasts. Sometimes this cytotoxic effect was neutralised by the addition of the patient's serum. In other cases addition of the patient's serum potentiated the cytotoxic effect and in occasional cases the cytotoxic effect was found to be dependent on the presence of the patient's serum.

"Natural" Antibodies and Anti-Tissue Autoantibodies

Sterzl and Silverstein (1967) considered that the term "natural antibody" should be reserved for those immunoglobulins which are formed spontaneously without antigenic stimulation. However, Wagner (1959) found that anti-bacterial agglutinins could not be detected in the sera of germ free rats and chickens and Springer, Horton, & Forbes (1959) demonstrated that naturally occurring anti-blood group B agglutinins in chickens were induced by antigens of E coli O_{86}. Similarly, Sterzl, Mandel, Miler & Riha, (1965) observed that, in contrast to the sera of conventionally reared piglets, the sera of precolostral germ-free piglets fed on non-antigenic diets contained no detectable antibodies reactive to a wide range of antigenic material. Thus, the majority, if not all the detectable antibodies in the sera

of normal animals are antigen induced. However, in the immunological literature the term "natural antibody" is conventionally taken to mean any demonstrable antibody, capable of reacting with an antigen, which is present in the body fluid of an animal which has not been artifically immunised. Although this usage is in many ways unsatisfactory it has the advantage that it does not presuppose any particular origin or function of natural antibodies. For this reason the conventional interpretation of the term "natural antibody" will be followed.

There is some evidence that the level of natural antibodies in the serum of normal animals is governed by genetic factors. Stern & Davidsohn (1954,a) found considerable differences in the levels of natural antibodies to sheep and chicken erythrocytes in inbred strains of mice. The possibility that these differences were due to bacterial infection was thought unlikely as the animals were housed together and these differences were shown not to be due to the presence or absence of tissue antigens shared with sheep erythrocytes (Stern & Davidsohn, 1956). Studies on the sera of hybrid mice and hybrid mice back-crossed with the original strain, supported the assumption that these levels were genetically determined (Stern & Davidsohn, 1954,a). The levels of anti-sheep and anti-chicken haemagglutinins were found to be independent of each other in the different strains of mice and were found not to be correlated with the ability of the mice to respond immunologically to the appropriate antigenic stimulation. This latter

finding was strikingly confirmed by Biozzi et al, (1968) who showed that there was no relationship between the magnitude of the immune response to the injection of sheep erythrocytes and the number of spleen cells forming natural anti-sheep erythrocyte antibody in mice.

It has been stated that a simple way to differentiate between normal and immune antibodies is to measure their respective heat labilities (Skarnes & Watson, 1957). Thus natural antibodies were supposed to be inactivated by heating at 56°C for 30 mins., whereas immune antibodies were supposed to be unaffected. Unfortunately there are a number of reports of heat stable natural antibodies e.g. bacterial agglutinins in the sera of cattle, horses and monkeys (Jordan, 1937) and anti-phage antibodies in normal human sera (Toussaint & Muschel, 1962). Moreover, some immune antibodies appear by certain tests to be heat labile e.g. anti-bacterial and viral antibodies in fowl sera as judged by complement fixation (Bushnell & Hudson, 1927; Rice, 1947; Brumfield & Pomeroy, 1957).

Evidence has accumulated over the years that antibodies, capable of reacting with autologous tissue antigens, are present in the sera of normal animals. An early report was that of Friedenreich (1929) who noted that normal human sera were able to agglutinate human erythrocytes treated with bacterial enzymes. Kidd & Friedewald (1942 a,b) described a heat stable (56°C for 30 mins.) complement fixing factor in the sera of normal rabbits,

which combined specifically with sedimentable tissue components. The factor was destroyed by heating at 65°C for 30 mins. and was precipitable by ammonium sulphate. It was found to react weakly with heated tissue extracts and not at all with alcoholic extracts. On the basis of these results, Kidd & Friedewald suggested that the factor was a natural antibody. These results were confirmed (Muschel, Simonton, Wells & Fife, 1961; Asherson & Dumonde, 1962) and extended by Asherson & Dumonde (1963) and Asherson & Rose (1963) who showed that the natural antibody behaved as a macroglobulin on DEAE chromatography and zone ultracentrifugation.

Asherson & Rose (1963) found a higher level of this natural antibody in rabbits infected with Eimera stieda, the causative organism of hepatic coccidiosis. On the basis of this finding they postulated that the stimulus to the production and maintenance of the Kidd-Friedewald antibody was bacterial. This hypothesis received support from the finding of autoantibodies, to a mucopolysaccharide antigen in the colon, ileum and stomach of rabbits, following the injection of various bacteria in Freunds' complete adjuvant (Asherson & Holborow, 1966).

Kershaw (1949) suggested that the appearance of natural antibodies in the serum of animals early in life was a reflection of physiological maturation. This idea was revived with respect to anti-tissue autoantibodies by Hook, Toussaint, Simonton & Muschel (1966) who described the simultaneous appearance of natural antibodies to micro-organisms and autologous tissue antigens (the

Kidd-Friedewald antibody) about 50 days after birth in rabbits.

A "Physiological" Mechanism

The demonstration that animals are able to react immunologically against their own effete tissue material led a number of immunologists to re-examine what Ehrlich intended by his concept of a "horror autotoxicus of the organism". In fact Ehrlich (On haemolysins: 3rd communication, Ehrlich, collected papers, 1957) suggested that immunological processes might be involved in reactions to "the absorption, by an animal, of its own cell material". Coombs (1958) considered that it was "Ehrlich who originally conceived the possibility of autoantibody production being a physiological method by which the body protected itself against autointoxication". Hence Coombs postulated that the physiogenic and pathogenic consequences of immune reactions are not a reflection of essentially different processes and thus if a physiogenic consequence of an immune reaction is exaggerated it may become inimical to the host.

Grabar (1957, 1959, 1965) was struck by the possibility that some endogenous material, such as products of cell destruction, may be recognised as foreign by the antibody forming system. He proposed that the immunoglobulins were a particular case of general physiological mechanism capable of uniting with various substances and serving as "carriers" or transporters of them. Autoantibodies were presumed to be carriers of catabolic products. He considered

that high levels of autoantibodies would represent a pathological exaggeration of a normal physiological mechanism.

Boyden (1963, 1966) too concluded that a degree of auto-immunity to certain intracellular components was a normal state. To him it seemed axiomatic that a metazoan system could not have evolved in the absence of some mechanism capable of rejecting foreign material. Thus he proposed that the mechanism by which the immunological system of vertebrates recognises foreign material evolved from that which enabled the phagocytic cells of simple animals to discriminate between indigenous and foreign material. As the phagocytic system of simple animals treats foreign bodies and damaged cells similarly then it would be expected that vertebrates would be able to respond immunologically to effete material and damaged cells. The events which follow the injection of antigenic material into the skin of an immunised animal are very similar to those which follow injury to the skin of a normal animal. Boyden suggested that this similarity was due to the series of events which characterise the cellular response being set in motion, in both instances, by antigen-antibody reactions. Now, Boyden (1962) had established that complement fixing antigen-antibody reactions activated a factor in normal serum which had a direct chemotactic influence on polymorphonuclear leucocytes (PMN). Moreover, Hurley (1963, 1964) had demonstrated that both the <u>in vitro</u> and <u>in vivo</u> migration of PMN towards various tissue homogenates was dependent on the interaction

of normal serum components with the homogenates. This, as Boyden (1964) pointed out, raises the possibility that auto-antibodies are implicated in the removal of tissue breakdown products by rendering them attractive to PMN.

Anti-tissue Autoantibodies and Tissue damage

Autoantibodies directed against tissue components have been detected in various human disease states e.g. anti-heart antibodies in patients with myocardial infarction (Ehrenfeld, Gery & Davies, 1961), anti-skin antibodies following burn injury (Feoderov & Skurovich, 1962), anti-kidney antibodies in kidney disease (Kramer, Watt, Howe & Parish, 1961) and anti-liver antibodies in liver disease (Gajdusek, 1957; Cohen, Otha, Singer & Popper, 1960; Fraga, Toledo & Lima, 1964; Walker, Doniach, Roitt & Sherlock, 1965). Weir (1963, 1966) noted that a common feature associated with many of these observations was the presence of tissue damage preceding the formation of the anti-tissue antibody. He considered that although it is tempting to allocate a causal role to such antibodies there is no direct evidence that they are responsible for tissue damage. He had failed to detect the binding of anti-nuclear antibody to tissue components *in vivo* (Weir, 1962) and it seemed important to him to determine whether anti-tissue antibodies might be stimulated by cellular breakdown. Accordingly, he induced liver damage in rats by the injection of carbon tetrachloride (CCl_4) and tested

their serum for anti-liver antibodies (Weir, 1961, 1963). Soon after the injection of CCl_4 he found a serum factor, detectable by complement fixation, which reached a high titre on the fourth day after injection and then rapidly disappeared. The response was not detectable by immunodiffusion or passive cutaneous anaphylaxis and it was prevented by X-irradiation or splenectomy of rats prior to the injection of CCl_4. The factor was reactive with heat labile sedimentable components of autologous and homologous liver and was found to behave as a macroglobulin (Weir, 1964,a). On the basis of these results Weir (1963, 1966) considered that the factor was an anti-tissue autoantibody.

Further work showed that this response could be induced by the injection of other hepatoxic agents e.g. chloroform, dimethylnitrosamine and tannic acid (Weir, 1964 b, Elson, 1965). In addition it was found that the liver antigen released by these agents appeared in the circulation about six hours after injection, reached a maximum about 24 hours and then rapidly disappeared.

These results have been confirmed and extended by a number of workers. Arnason, Salomon and Grabar (1964) detected high levels of anti-liver autoantibody, by passive haemagglutination, in germ free mice following the injection of CCl_4. Sargent, Myers & Richter (1966) injected thioacetamide into rats and tested their sera for anti-liver antibodies at three day intervals. They detected a response in only 10 per cent of rats. However,

Weidermann, Reinhardt & Denk (1966) injected thioacetamide into rats and detected an IgM anti-liver autoantibody by latex agglutination and complement fixation. The antibody reached high levels four days after injection and then rapidly disappeared.

In the report of Weir, (1963) it was noted that some 15 per cent of normal rat sera contained an anti-liver autoantibody. A similar autoantibody, directed against kidney has been found in normal rat sera (Digby & Loewi, 1965). Moreover, the response described following the injection of CCl_4 had the features of a secondary response. These observations suggested that some anti-liver autoantibody might occur in the sera of all normal rats in response to the continual release of intracellular breakdown products from effete liver cells. Such antibody, would, however, only be detectable providing a sufficiently sensitive serological technique was used. This work describes the finding and properties of a serum factor, reactive with tissue homogenates, in the serum of most "normal" rats. The attempts to establish the antibody nature of this factor and to characterise the antigens with which it reacts, are reported. The results of these studies led to an investigation of the effects on the primary binding and complement fixing ability, of heating rat anti-sera. Other work was directed towards defining the role of the anti-tissue serum factor. Finally, attempts were made to determine what factors influence the ability of the tissue antigens, which react with the serum factor, to circumvent the "normal" tolerance inducing mechanisms.

MATERIALS AND METHODS

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Animals

Albino rats of both sexes, originally derived from the Wistar strain, were used. They were obtained from the Small Animal Breeding Station, Bush Farm, Midlothian. They were housed in wire cages and fed on standard rat cubes and water, both ad lib.

New Zealand white, Californian and outbred rabbits were used for routine production of anti-sera.

Outbred Guinea pigs provided sera which was used as a source of complement.

Bleeding

The method of bleeding employed for all animals was cardiac puncture. This was found to be an efficient method, particularly where large quantities of blood were required, whilst it caused the animal minimal physical discomfort. The technique was varied slightly according to the size and excitability of the animal being bled.

Rats were anaethetised under ether and blood withdrawn via a needle, inserted at about a 30° angle under the xiphoid process, with the syringe parallel to the midline. The heart was entered by advancing the needle forwards until the pulse could be felt and then giving the needle a quick thrust towards the direction of the pulse. After the required quantity of blood had been collected the needle was quickly withdrawn from the chest. The

size of needle used was considered important in bleeding. The use of a large needle seemed to be associated with increased fatalities although it facilitated speedy withdrawal of blood. For a rat a 23 G needle, for a guinea pig a 21 G needle and for a rabbit a 19 G needle were found most suitable.

Guinea pigs were bled as described above but rabbits were bled without anaesthesia being immobilised by securing them to a "rabbit board".

Collections of Serum

Blood was allowed to clot overnight at 4°C. The serum was removed using a Pasteur pipette and stored at -25°C. until required for use.

Techniques of Antibody Assay

(1) Complement Fixation

The test is based on two properties of complement:- (i) its capacity to combine irreversibly with some antigen - antibody complexes (ii) its ability to lyse sheep erythrocytes coated with a specific haemolysin (sensitised sheep erythrocytes). The test is performed by adding a known amount of complement to a mixture of serum and antigen. After allowing time for complement to be fixed, the residual complement is back-titrated by an indicator system consisting of sensitised sheep erythrocytes. If the serum contains antibody against the antigen used then the

complex formed will fix complement and none will be available to lyse the sheep erythrocytes. Lysis of the sheep erythrocytes indicates the absence of specific antibody in the serum.

The amount of complement fixed by a constant quantity of antigen and varying amounts of anti-serum was studied by Osler, Mayer & Heidelberger (1948) using type III pneumococcal polysaccharide and the appropriate anti-serum. It was shown to follow a curve consisting of two linear segments the break between which was near to the zone of equivalence as measured by quantitative precipitation. The steep segment, where the amount of complement fell rapidly with increasing anti-serum dilution, was beyond the zone of equivalence. Now the amount of complement required to cause lysis of a given number of sensitised sheep erythrocytes has been shown to follow a sigmoidal curve (Kabat & Mayer, 1961). Thus for precise measurement of haemolytic complement a 50% end point, where the degree of lysis is sensitive to small changes in the amount of complement, would be chosen. However, as the amount of complement fixed falls off rapidly beyond the zone of equivalence with increasing anti-serum dilution then beyond the zone of equivalence sufficient complement becomes available to lyse the sheep erythrocytes completely over one or two anti-serum dilutions. Consequently, where it is not essential to have a precise endpoint, and where it would be more convenient, the end point might be taken as the last serum dilution containing unlysed cells.

A procedure for assay of antibody activity by complement fixation was required which enabled large numbers of sera to be tested simultaneously and for which only small quantities of the test materials were available. A precise measurement of antibody was not essential.

Sensitisation of Sheep Erythrocytes

Sheep blood was collected directly from the abattoir and stored in an equal volume of sterile Alsever's solution at 4°C, for one week. After this period it was found that the erythrocytes were uniformly susceptible to lysis by complement and remained so for about three weeks. For the preparation of 100 ml. of 3 per cent sensitised erythrocytes about 15 ml. of the blood Alsever's mixture was required. It was centrifuged at 1000 G. for ten minutes and the supernate discarded. The erythrocytes were washed twice in 0.85 per cent saline and twice in veronal C.F.T. buffer pH 7.2 (Oxoid Ltd., London). 3 ml. of the washed packed sheep cells were suspended in 47 ml. C.F.T. buffer. For erythrocyte sensitisation, 0.6 ml., equivalent to 10 M.H.D. for the number of erythrocytes used, of Horse Haemolytic serum for Sheep Corpuscles (Burroughs Wellcome & Co., London) was used. It was diluted with 49.4 ml. C.F.T. buffer and then mixed slowly into the erythrocyte suspension. The mixture was agitated gently and incubated for thirty minutes in a 37°C. water bath to allow maximal sensitisation of the erythrocytes. The suspension

was stored at 4°C. and was used within four days. Prior to use the erythrocytes were resuspended by shaking gently and were allowed to warm to room temperature.

Materials.

Clean M.R.C. pattern perspex plates.

Oxoid C.F.T. buffer pH 7.2 as diluent.

Guinea-pig complement.

Sensitised sheep erythrocytes.

50 drop/ml. Pasteur pipettes (Harshaw Chemicals Ltd., Daventry).

Procedure.

The four-drop system, as described by Weir (1967), was used and carried out in M.R.C. pattern perspex plates. 1.25 M.H.D. of guinea-pig complement was determined by its titration in the presence of antigen. A "checkerboard" titration of antigen against a known positive anti-serum was performed to select the antigen concentration which gave optimal complement fixation over a range of anti-serum dilutions. A doubling dilution series of the test serum was made in Oxoid C.F.T. buffer. Starting from the highest dilution, one drop of each dilution was transferred via a straight ended Pasteur pipette (50 drop/ml. commercially produced Pasteur pipettes were found suitable) to the wells of a perspex plate. To this test series one drop of complement and one drop of antigen were added from an identical pipette. Antibody controls with two drops of serum and one of complement were set up and antigen controls, containing no serum, but one or two

drops of antigen, one drop of complement and buffer to make up the volume (see table). These controls are such that any anti-complementary effect of either the anti-serum or antigen will show up quite clearly. In practice if the antigen control showed anti-complementary activity then the test result was ignored and if the serum controls showed anti-complementary activity the test result was recorded as anti-complementary.

	Test Series	Antibody Control	Antigen Controls
Anti-serum dilution (from doubling dilution series)	1 drop	2 drops	
Complement (1.25 MHD)	1 drop	1 drop	1 drop
Antigen	1 drop		1 drop + 1 drop buffer 2 drops

As the test materials are heat labile they were kept in ice baths so as to maintain their temperature at 0°C. The perspex plates rested on ice whilst the tests were set up.

The tests were incubated at 37°C. for thirty minutes to allow complement to be fixed. One drop of three per cent sensitised sheep cells was added to each well and the test read after a further forty minutes incubation after having been carefully shaken at 20 minutes. The end point was taken as the last well showing clearly visible red cells.

"Micro" - complement Fixation Test

Where a precise estimation of the complement fixing activity of a serum was required a modification of the "micro" complement fixation test described by Levine (1967) was used.

Procedure.

Two parallel 1.0 ml. doubling dilutions of the test serum in Oxoid CFT buffer (pH 7.2) were set up in Wassermann tubes at 0-4°C. To each tube was added 1.0 ml. 3 MHD guinea pig complement and to the test series 1.0 ml. of the antigen solution. 1.0 ml. buffer was added to the antibody control series and antigen controls were set up containing 2.0 ml. of the antigen solution and 1.0 ml. complement. The tubes were incubated at 37°C. for thirty minutes. 1.0 ml. 0.5 per cent sensitised sheep erythrocytes were then added to each tube and they were incubated for a further forty minutes at 37°C. After immersion in an ice bath, to stop the haemolytic reaction, the tubes were centrifuged for 10,000 g. mins. at 4°C. to sediment unlysed erythrocytes. The supernates were removed, allowed to warm to room temperature, and their optical densities (OD) measured at 541 μ in a Unicam SP 500 spectrophotometer. The OD of the supernates was plotted against the reciprocal of the anti-serum dilution. The reciprocal anti-serum dilution at which fifty per cent of the added sensitised cells were lysed was found from the graph by interpolation and taken as the end point.

Radio-iodination of Bovine Serum Albumin

Proteins can be trace labelled by substituting radio-iodine in their tyrosine residues. Iodide is oxidised by a suitable oxidising agent in the presence of protein. Cationic iodine (I^+) is formed which in alkaline solution, substitutes predominantly in the meta position of tyrosine groups. Iodination of the other groups, e.g. tryptophan, can occur but in the case of bovine serum albumin (BSA) it is thought to take place exclusively in the tyrosine group (Hunter, 1967). In the method of Hunter & Greenwood (1962) the oxidising agent used is hypochlorous acid, formed by the slow dissociation of Chloramine T in aqueous solution. By this method small quantities of protein can be labelled with high efficiency without recognisably altering the protein (Hunter, 1967).

Materials.

Borate buffer pH 8.4 ionic strength 0.1.

1-4 m.c. Carrier free sodium iodide (NaI^{131}) free of reducing agent (purchased from Radiochemical Centre, Amersham, Bucks.).

Potassium iodide (KI).

Chloramine T (sodium p-toluenesulphonchloramide) 50 mg/100 ml. distilled water.

BSA solution: 20 mg/ml.

Procedure.

The Chloramine T method as described by Hunter (1967) was

used. The iodination reaction was performed in a fume cupboard behind lead shielding. A small reaction flask containing 1-4 m.c. carrier free iodine^{131} was placed on a magnetic stirrer. 0.2 ml. borate buffer and 0.2 ml. BSA solution were added followed immediately by 0.2 ml. of the Chloramine T solution. The mixture was stirred and allowed to react for sixty seconds. 4 ml. of borate buffer was added and the mixture transferred via a hypodermic syringe to a dialysis sac. The mixture was dialysed against numerous changes of borate buffer, to remove the unbound iodine, until the radio-activity of the dialysate remained constant. The concentration of I^{131}-BSA in µgN/ml. was determined by comparing the preparation spectrophotometrically with a standard of known concentration. The I^{131}-BSA preparation was only used if greater than 99 per cent of the radio-activity was precipitable by ten per cent trichloracetic acid.

(2) Ammonium Sulphate Precipitation Test (Farr Technique)

Many antibody assays measure a secondary effect of the primary antigen-antibody union, e.g. the capacity of an antiserum to fix complement. The effect selected may not reflect the total antibody content of the antiserum since antibodies are heterogeneous with respect to the secondary effects they produce. A measure of the primary interaction of antigen and antibody is therefore necessary to assay the total antibody content of an antiserum.

The Farr technique depends on the differential solubility of

antigen and antigen-antibody complexes in 50 per cent saturated ammonium sulphate. It is limited to antigens which are soluble in 50 per cent saturated ammonium sulphate. Antibody bound antigen is precipitated by 50 per cent ammonium sulphate due to the insolubility of the bound globulin. The unbound antigen remains in solution. At equilibrium the reaction between antigen and antibody can be represented as shown in the equation:-

$$\text{Antigen} + \text{Antibody} \underset{Kd}{\overset{Ka}{\rightleftharpoons}} \text{Antigen-Antibody}$$

where Ka = association constant

Kd = dissociation constant

50 per cent saturated ammonium sulphate has been shown to effectively freeze this equilibrium (Farr, 1958) by preventing formation and dissociation of the complex. The amount of antigen in the precipitate is thus a close approximation to the amount of antigen bound at equilibrium in solution.

The fate of the antigen can be followed by using radioactive labelled antigen. In the case of I^{131} labelled bovine serum albumin, as used in this procedure, the labelling has been shown not to interfere with its immunological properties (Farr, 1958).

The test is performed by adding constant amounts of labelled antigen to serial dilutions of the antiserum. After allowing time for equilibrium to be established an equal volume of saturated ammonium sulphate is added. The resultant precipitate is centrifuged and washed with 50 per cent saturated ammonium sulphate.

The number of radioactive disintegrations/unit time emitted by the precipitate are counted and the percentage of antigen bound, i.e. precipitated with the globulin, at each antiserum dilution is determined. The percentage of antigen bound is plotted against the log reciprocal antiserum dilution. From this, the antiserum dilution which would bind 33 per cent of the added antigen can be determined. This value is used to calculate the amount of antigen bound/ml. serum. It is expressed in μgN bound/ml. undiluted serum at a given antigen concentration and is designated the antigen binding capacity at the 33 per cent end point (ABC 33).

Material.

Borate buffer pH 8.4 ionic strength 0.1.

6.184 gm boric acid.

9.536 gm sodium tetraborate.

4.384 gm sodium chloride.

Made up to 1 litre with distilled water.

Saturated ammonium sulphate (SAS) specific gravity 1.240 at 4°C.

Half saturated ammonium sulphate (SAS/2). One volume of SAS to one volume borate buffer.

Normal rat serum diluted with borate buffer.

20% Trichloracetic acid in aqueous solution (TCA).

Trace labelled I^{131} Bovine serum albumin (BSA*).

The stock solution was diluted with 1/100 normal

serum to give the required dilution.

Procedure.

The procedure followed for determining the antigen binding capacity of an antiserum was based upon the method described by Farr (1958) and Minden & Farr (1967). Serial dilutions of the antisera were made using volumetric blow-out pipettes. The sera were usually diluted 1/10 in borate buffer and subsequent dilutions made in 1/10 normal rat serum. With antisera taken early in a primary response 1/6 dilutions were made initially and doubling dilutions made in 1/6 normal rat serum. Starting with the highest dilution 0.50 ml. amounts of each antiserum dilution were transferred to a series of clean Wasserman tubes. This experimental series was set up in duplicate for each antigen concentration used. This and all other steps in the procedure was carried out at 0-4°C.

Groups of control tubes (see table) were set up to determine:-

(i) the total amount of radioactivity added/tube. This group, Ag Ad, was to receive antigen and no further treatment;

(ii) the total amount of precipitable radioactivity (i.e. protein bound I*)/tube. Each tube in this group (TCA) received 0.50 ml. of 1/10 normal rat serum. These tubes were to receive 20 per cent TCA.

(iii) the amount of non specifically bound BSA*/tube. Each tube in this group (C) received 0.50 ml. 1/10 normal rat serum and was to receive ammonium sulphate. These

controls were repeated for each antigen concentration used.

0.5 ml. of the BSA* preparation was added to each of the experimental and control tubes using a 1 ml. automatic syringe with a valve and canula attachment (R.B. Turner & Co. Ltd., London). The concentration of BSA*/ml. used in a particular experiment depended on the expected antigen binding capacity (ABC) of the antiserum. Where high ABC's were expected 0.2 μg N BSA*/ml. was used and where low ABC's were expected 0.02 μgN BSA*/ml. was used. To detect small quantities of anti BSA antibody, e.g. in serum fractions, 0.01 μgN BSA*/ml. was used.

After addition of BSA* the tubes were incubated at 4°C. overnight (approximately 16 hours). 1 ml. SAS was then added, with an automatic syringe, to each of the experimental and control tubes except the Ag Ad. Each tube was mixed vigorously on a vibrator immediately after the addition of SAS. They were allowed to stand at 0-4°C. for 30 minutes and then centrifuged at 1500 G for 30 minutes in an M.S.E. "major"-refrigerated centrifuge (4°C.). The supernates were decanted and discarded and the tubes blotted on absorbent paper to remove any remaining drops of supernate. The precipitates were resuspended in SAS/2 and, after incubating at 0-4°C. for 30 minutes, the tubes were again centrifuged at 1500 G for 30 minutes. 1.0 ml. Trichloracetic acid was added to each of the TCA control tubes. They were incubated at 0-4°C. for 30 minutes, centrifuged at 1500 G

for 30 minutes and the supernate discarded.

Tube	Serum	BSA* preparation	Precipitated with	to determine
Experimental Exp	0.50ml. antiserum	0.5ml.	SAS	amount of added BSA* bound
Controls Ag Ad		0.5ml.		total amount of added radioactivity
TCA	0.50ml. normal serum	0.5ml.	20% TCA	total amount of precipitable radioactivity
C	0.50ml. normal serum	0.5ml.	SAS	amount of non specifically bound BSA*

The number of radioactive disintegrations/unit time in each tube was counted in a Nuclear Enterprises "Gamma-matic" scintillation spectrometer Mark 1 A with a two inch well type sodium iodide crystal. The analyser window of the counter was set to count emissions with a quantum energy of 0.364 MeV. The counting period was adjusted so that the probable counting error of each experimental tube containing above 20 per cent BSA* bound did not exceed 2 per cent. As the disintegrations are isolated events in time (i.e. follow a Poisson distribution) then:-

$$\text{The probable counting error} = \sqrt{\text{number of counts}} \times \frac{100}{\text{number of counts}}$$

Thus if there are 10,000 counts, then the probable counting

error is 1 per cent and the maximum counting error is 3 per cent.

It was necessary to check that the I^{131} was bound to BSA. In the TCA control all the bound I^{131} was precipitated with the protein whereas any unbound I^{131} was discarded with the supernate. Thus:-

$$\text{the \% protein bound iodine} = \frac{\text{counts/unit time in TCA}}{\text{counts/unit time in Ag Ad}} \times 100$$

In all experiments, the value was between 99 - 100 per cent. If it had been less than 99 per cent then the BSA* preparation would have been discarded.

Calculating Procedure.

For each of the control groups viz. C, TCA and Ag Ad, the average number of counts was determined. The number of counts in each of the duplicate experimental tubes was averaged. The percentage of the total added BSA* bound specifically at each antiserum dilution was calculated by substituting in the formula:-

$$\text{\% BSA* bound} = \frac{\text{Counts in Exp - Counts in C}}{\text{Counts in TCA - Counts in C}} \times 100$$

The percentage BSA* bound was plotted against the reciprocal of the antiserum dilution on semilogarithmic graph paper with the percentage BSA* bound on the linear axis and the reciprocal of the antiserum dilution on the log axis. The best straight line was drawn between points where the percentage bound was less than 85 per cent but more than 15 per cent. The reciprocal antiserum dilution which would bind 33 per cent of the total added antigen

(reciprocal end point dilution) was found from the graph by interpolation.

The antigen binding capacity at the 33 per cent end point (ABC - 33) of the antiserum was given by:-

ABC - 33 at the particular concentration used = Reciprocal end point dilution x ugN BSA* x 2 x .33

(3) Immunoelectrophoretic Analysis

This technique was used as a qualitative measure of the uptake of antibody by particulate tissue antigens. If an antigen is mixed with its specific antiserum then various serum components become bound to the antigen. An antiserum against these serum components can be raised by injecting the washed immune complex into a heterologous animal. This antiserum can then be used to characterize the serum components in the complex.

This principle was used to determine whether a rat mitochondrial preparation (F_3) would bind any components of normal rat serum. If there is an antigen antibody reaction between F_3 and normal rat serum then immunoglobulins and complement components should become bound to the F_3. An antiserum can be raised against any rat serum components which bind to F_3 by injecting F_3 treated with normal rat serum into an animal from another species. This antiserum can then be analysed for anti-rat serum components.

Materials.

Mitochondrial Preparation of rat liver (F_3).

Rat serum pool.

0.85% saline.

Microscope slides (3 x 1 ins.).

0.85% agar in distilled water

0.85% agar in veronal buffer.

pH 8.6. ionic strength 0.025 (2.797 gm/ 1 Barbituric acid, 20.6 gm/ 1 sodium barbital, diluted 1/4).

Shandon cutter.

Shandon electrophoresis cell.

Amido (napthalene) black: 400 mg. /litre in acid methanol.

Acid methanol: 450 ml. methanol, 450 ml. deionized water and 100 ml. acetic acid.

Procedure.

Preparation of antisera.

Two hundred and fifty milligrams wet weight of a mitochondrial preparation (F_3) of rat liver in 3 ml. 0.85% saline was incubated with 3 ml. normal rat sera at room temperature for 30 minutes and overnight at 4°C. F_3 was treated similarly with decomplemented normal rat sera. The mixtures were centrifuged at 9000 G for 10 minutes and the supernates discarded. The pellets were washed three times and resuspended in 0.85% saline. These suspensions and a suspension of washed F_3 were injected into rabbits. Each rabbit received one of the three suspensions

intravenously and the same suspension emulsified with Freund's complete adjuvant subcutaneously. The rabbits were boosted after thirty days and bled seven days later. Antisera against F_3 exposed to a number of rat serum preparations were prepared similarly in rabbits and guinea pigs. The antisera were stored at -25°C. until required for testing.

Analysis of antisera by Immunoelectrophoresis.

The micro-immunoelectrophoretic technique of Scheidegger (1955) was used. Microscope slides were washed in boiling pyroneg solution to remove grease and rinsed thoroughly. They were coated with 2 ml. 0.85% agar in distilled water and dried. The coated slides were placed horizontally on a level (Shandon Scientific Co.) and 2 ml. of 0.85% agar in buffer at 85°C. was applied. The agar gel thus formed was found to adhere firmly to the coated slides. A Shandon cutter was used to punch out troughs and wells in the agar. The agar in the wells was removed. The slides were placed on a rack which was put in position in an electrophoresis cell. The wells were filled with rat sera or serum fractions which were electrophoresed for 100 minutes employing a potential gradient of 5-6 V/cm. on the slide. After electrophoresis the agar in the troughs was removed and the troughs filled with antisera prepared as described above. The slides were placed in a humid atmosphere at room temperature to allow development of precipitin bands. The slides were washed in 0.85% saline (48 hours) and distilled water

(48 hours) to remove unbound protein. They were stained in amido black (15 minutes) which stains the remaining protein and washed in acid methanol (48 hours) to remove the unbound stain. The slides were dried and examined for precipitin bands.

(4) Radioimmunoelectrophoresis

This method provides a qualitative measure of the primary interaction of antigen and antibody. The antiserum to be tested is first electrophoresed. It is then precipitated with a suitable antiglobulin serum and allowed to react with radioactive labelled antigen. Unbound antigen is washed away. The distribution of any remaining radioactivity (i.e. bound antigen) is examined using an X-ray film.

Procedure.

a. Indirect.

Rat anti-BSA sera were electrophoresed using the micro-immunoelectrophoretic technique described above. After electrophoresis a rabbit anti-rat immunoglobulin was run into the troughs and the precipitin bands allowed to develop for 48 hours. The slides were washed for 48 hours to remove unprecipitated protein. The troughs were then filled with a BSA* solution containing 1 ugN BSA/ml. and approximately 1.5 $\mu c I^{131}/\mu gN$. After 24 hours the slides were washed for 48 hours in 0.85% saline to remove unbound BSA. The slides were air dried and overlaid with Kodirex X-ray film for 24-48 hours. The film was developed and

examined.

b. Direct.

Rat anti-BSA sera were electrophoresed and a mixture of I^{131}BSA and the precipitating anti-rat globulin was added to the trough. The slides were then treated as described above.

Serum Fractionation Procedures

1. Gel Filtration (Exclusion) Chromatography

Proteins can be separated according to their molecular dimensions by chromatography on a column of a gel consisting of beads of a cross linked dextran commercially available as Sephadex (Tiselius, Porath and Albertsson, 1963). For the fractionation of serum proteins Sephadex G-200, with an exclusion limit of molecular weight about 200,000 is used. The pore size of the beads is such that proteins of molecular weight greater than 200,000 (e.g. IgM) are excluded from the beads and pass through the column unhindered with the void volume. Proteins of molecular weight smaller than 200,000 are able to enter the beads and hence their progress through the column is retarded. The number of molecules which enter the beads determines the rate of progress of a particular molecule through the column (Fahey & Terry, 1967). Consequently, proteins of intermediate molecular weight (e.g. IgG) are eluted before proteins of lower molecular weight (e.g. albumins).

Materials.

Sephadex G-200.

0.1 M Tris-HCl 0.2 M NaCl adjusted to pH 8.0.

Column.

L.K.B. peristaltic pump.
L.K.B. Uvicord Automatic Scanning device.
L.K.B. radirac fraction collector.

Procedure.

The procedure was similar to that recommended by Fahey & Terry (1967). Sephadex G-200 (Pharmicia Fine Chemicals, Uppsala, Sweden) was allowed to swell for a week in excess 0.1 M tris 0.2 M NaCl buffer adjusted to pH 8.0. Fine particles were removed by periodically decanting the supernatant fluid and mixing in more buffer. The resultant slurry was deaerated and poured into a column. The outflow tap was opened and additonal slurry added until the column was full. Buffer was passed through the column overnight to allow the beads to settle. With the outflow tap closed, the serum sample was layered carefully over the gel surface using a glass hypodermic syringe. The column was connected to a reservoir of buffer about 10cm. above the column. The outflow tap was opened and buffer allowed to run through the column at a constant flow rate, the outflow being controlled by an L.K.B. peristaltic pump. The eluate was passed through an L.K.B. Uvicord automatic scanning device, which measured the percentage transmission at 280mμ, and a tracing of the protein distribution was obtained. Fractions of 8 ml. volume were collected on an L.K.B. radirac fraction collector. To obtain adequate resolution of large serum samples a large column (6 x 80 cm.) was used. Samples of not more than 2 ml. were well separated on a 2.5 x 35 cm. column.

The distribution of individual human serum components after fractionation on Sephadex G-200 has been studied using immuno-electrophoretic analysis, double gel diffusion using specific antisera and analytical ultracentrifugation (Flodin and Killander, 1962; Fireman, Vannier & Goodman, 1964). Three major protein peaks occurred in the effluent. The first peak contained IgM and $\alpha 2$ macroglobulins, the second contained mainly globulins and the third mainly albumins.

2. Zone Centrifugation in Sucrose Density Gradients

Kunkel (1960) was the first to describe a method for the separation of serum proteins by centrifugation on sucrose density gradients. For a particular protein in a centrifugal field the velocity of sedimentation = $W^2 (p - s) \frac{x}{r} \times S$.

where w = the angular velocity

p = density of protein

s = density of medium

x = distance from the axis of rotation

r = viscosity of medium

and S = the sedimentation constant

In an experiment at constant w and temperature the buoyancy terms (p - s), for ordinary proteins in not too strong sucrose is nearly independent of position (Charlwood, 1966). Furthermore, r is nearly proportional to x. Thus for a particular protein the velocity of sedimentation depends mainly on the sedimentation constant. Since for serum proteins their sedimentation constants

are nearly proportional to their molecular weights (Stanworth, 1967) then they are separated according to their molecular weights.

The use of a sucrose density gradient is to stabilize the moving zone by preventing radial convection. The gradient has the effect of directing convection currents across the centrifugal field. The combination of increasing viscosity and density of the sucrose solution making up the gradient also results in the progressive retardation of faster sedimenting molecules, e.g. IgM.

Materials.

Polypropylene centrifuge tubes (5c.m. x 1.1c.m.)

Solutions of 10, 20, 30 + 40% sucrose made up in phosphate buffered saline pH 7.0.

M.S.E. Superspeed 40 centrifuge, with temperature control of the rotor.

Procedure.

Sucrose density gradients were prepared by layering 1 ml. of 30, 20 and 10% sucrose solutions respectively over 1 ml. of 40% sucrose solution in centrifuge tubes, taking care that the meniscus between each solution was not broken. Adhesive tape was placed over the top of the tubes to prevent evaporation. The layers were allowed to equilibrate for 24 hours at 4°C.

which results in the production of a linear gradient from 12% sucrose solution at the top of the tube to 37% at the bottom (after Kunkel, 1960). 0.5ml. of the serum sample, diluted 1/2 with phosphate buffered saline pH 7.0. was layered on top of the gradient. The meniscus thus formed was blurred with a bent loop, to prevent instability at this boundary giving rise to "droplet" formation or streaming of the sample. Tubes were placed in a three place swing out rotor and were spun at 35,000 r.p.m. for 16 hours at 4°C. in an M.S.E. 40 superspeed centrifuge. After centrifugation nine fractions of 0.5 ml. were taken from the top of each tube using a 1 ml. tuberculin syringe. The syringe was held just below the meniscus and gently screwed up so that the fraction of lowest density was removed first. The fractions were stored at - 25°C. until required for use.

Weir et al (1966) standardised this technique by fractionating early primary rabbit anti-bovine serum albumin (BSA) serum. Two peaks of antibody activity were found when the fractions were tested by passive haemagglutination and the Farr test. The more slowly sedimenting peak (IgG) was found in fractions 3-4 and the more rapidly sedimenting high molecular weight peak was found in fractions 6-8 (IgM). Chicken anti-BSA serum taken 6 days after primary injection, which is known to contain its main haemagglutinating activity in the IgM class (Dreesman et al, 1965) was also found to have its main haemagglutinating activity in fractions

6-8.

3. Salt Fractionation

Most proteins are precipitated out of aqueous solution by addition of high concentrations of neutral salt. The salt acts by hindering the interaction of the charged polar groups of the protein with water molecules. This favours an interaction between neighbouring protein molecules thus decreasing the solubility of the protein.

Materials.

Normal rat serum pool

Hydrated Sodium sulphate ($Na_2 SO_4 10.H_2O$)

Procedure.

Hydrated Sodium sulphate (34gm./100ml. sera) was added slowly, with constant stirring to a serum pool. The mixture was stirred for 15 minutes after a precipitate had been formed. It was centrifuged for 30,000 g minutes and the supernate discarded. The precipitate was dissolved in 0.02 M phosphate buffer pH 6.6. and dialysed overnight against this buffer. This procedure was used to prepare a crude γ globulin fraction from serum prior to further purification procedures.

4. Preparation of IgG using an Anion Exchange Resin

The use of an anion exchange resin such as diethylaminoethyl (DEAE) cellulose provides a simple procedure for the preparation of purified serum IgG (Fahey & Terry, 1967). DEAE is suspended in a buffer of about neutral pH and low ionic strength and serum added. Under these conditions negatively charged serum proteins adsorb onto the cellulose by electrostatic binding, the negatively charged groups on the protein interacting with positively charged groups on the cellulose. Only the noncharged serum proteins viz. IgG, are not adsorbed and these remain in solution in the suspending medium.

Preparation of DEAE cellulose.

DEAE cellulose was placed in a large beaker and treated as follows:-

(1) stirred for 24 hours in N sodium hydroxide.

(2) stirred for 24 hours in N hydrochloric acid.

(3) stirred for 24 hours in N sodium hydroxide.

The supernates from each treatment were discarded by decantation which also served to remove fine particles. The cellulose slurry was washed repeatedly in deionized water until the pH of the supernate approached 7. The slurry was then placed in 0.02 M buffer pH 6.6. and allowed to stand for 2-3 hours. The supernate was decanted and discarded and more buffer added. This process was repeated until the pH of the supernate was 6.6.

Batch Process.

This was used to purify crude γ globulin fractions prepared by salt fractionation. The crude fraction was dialysed overnight in 0.02 M phosphate buffer pH 6.6. The dialysate was stirred into a suspension of DEAE (about 1 gm/ml. serum added) in 0.02 M phosphate buffer pH. 6.6 and allowed to stand for 10 minutes. The resultant sludge was filtered under pressure through a sintered glass filter. The filtrate was mixed into fresh DEAE and, after standing for 10 minutes, again filtered through a sintered glass filter. The IgG contained in the filtrate was concentrated by ultrafiltration.

Column Chromatography.

Highly purified IgG was prepared from a crude γ globulin preparation by chromatography on a column of DEAE cellulose. The crude γ globulin preparation was dialysed overnight against 0.02 M phosphate buffer pH 6.6. The dialysate was centrifuged to remove insoluble material and the supernate applied to the column. Buffer (0.02 M phosphate pH 6.6.) was allowed to run through the column at a constant flow rate (controlled by an L.K.B. peristaltic pump). The protein content of the eluate was monitered on an L.K.B. Uvicord automatic scanning device and fractions of 8 ml. volume collected on an L.K.B. radiac fraction collector. The protein containing fractions were pooled and concentrated by ultrafiltration. The resultant preparation was tested by immuno-

electrophoretic analysis and found to contain only IgG.

Preparation of Tissue Antigens

For the preparation and separation of antigenic sub-cellular material it is first necessary to disrupt the cells of the tissue without damaging the intracellular organelles. Shear forces, produced by rotating a cylindrical plunger with a spherical end in a thick walled glass tube of slightly larger diameter, have been commonly employed. The tissue is suspended in a suitable medium and homogenised by moving the rotating plunger up and down the tube (Potter & Elvehjem, 1936). The choice of medium is an important consideration. 0.88 M sucrose preserves the morphological appearance of mitochondria whilst 0.25 M sucrose preserves their biochemical activity. The addition of various salts is beneficial to the preservation of some sub-cellular particles but deleterious to others (Mathias, 1966).

One of the main problems in attempting to separate tissue antigen is their extreme lability. Pinckard and Weir (1966) found a 50 per cent loss in antigenic activity after incubating a mitochondrial preparation of rat liver at 37°C. for 30 minutes. Differential centrifugation has the advantage that it can be carried out rapidly at 0-4°C. in an isotonic medium. The separation of sub-cellular particles is made according to their size and density. A tissue or organ homogenate is centrifuged until the faster sedimenting particles have formed a pellet. The

supernate is taken off and centrifuged further and the pellet resuspended in medium. The pellet will inevitably be contaminated with slower sedimenting particles and the supernate with fragments of faster sedimenting particles. The contents of the resultant fractions have been investigated by electron microscopy (Horne & Whittaker, 1962) and by the location of enzymes characteristic of particular organelles, e.g. succinic dehydrogenase in mitochondria (Hogeboom, Schneider and Pallade, 1948).

Materials.

0.25 sucrose.

Potter-Elvehjem homogeniser fitted with a Teflon pestle.

M.S.E. fractional horsepower variable speed motor.

10 ml. syringe with a long needle attached.

Procedure.

The modification of Schneider and Hogeboom's (1950) procedure described by Pinckard & Weir (1966) was used. Rats were fasted for 12-18 hours to lower the glycogen content of the liver. They were killed by stunning and bled. Their livers and other organs if required were immediately removed and chilled by washing in 0.25 M sucrose. This and all other fractionation steps were carried out at 0-4°C. The tissue was cut into small pieces with scissors and any fibrous connective tissue removed. If the organ or tissue was fibrous e.g. heart, further mincing in a

Waring blender was found to facilitate homogenisation. Three volumes of 0.25 M sucrose was added to the chopped or minced tissue. Homogenisation was carried out in a Potter-Elvehjem smooth walled glass tube with a fitted Teflon pestle rotating at 1200 r.p.m. The pestle was attached to an MSE fractional horsepower variable speed motor. The resultant 1/4 homogenate was filtered through muslin to remove any unbroken tissue. It was stored at -25°C. until required for use.

Fractionation of the homogenate by differential centrifugation was carried out immediately after its preparation. The homogenate was centrifuged at 1000 G for 10 minutes in a refrigerated centrifuge and the supernate removed by means of a syringe and long needle. The pellet was resuspended in 1 volume of 0.25 M sucrose and centrifuged for 6000 G minutes. The supernate was removed and the pellet resuspended in 0.25 M sucrose to a volume equal to that of the original homogenate making a 1.4 nuclear extract (F_1). The nuclear supernates were combined and fractions prepared from the pellets obtained by centrifugation for the following G minutes.

Fraction	G.min.	Content
2	30,000	mitochondria
3	90,000	mitochondria
4	150,000	mitochondria
5	260,000	mitochondria
6	6,000,000	microsomes
7		cell sap

The pellets were resuspended in 0.25 M sucrose to a volume equal to that of the original homogenate. The final supernate was taken as F_7.

Pinckard & Weir (1966) prepared liver fractions by this procedure and examined their content by electron microscopy. They labelled the fractions as shown above.

Estimation of Polymorph Migration

Various techniques have been evolved which purport to measure the migration of leucocytes.

In the capillary tube method the test material is drawn into a short capillary tube and the tube placed in a suspension of leucocytes. Active migration of the leucocytes is judged to have taken place if an accumulation of leucocytes is found in the tube. The validity of this method was questioned as early as 1898 (see review by Harris, 1961). The slide technique in which the migration of leucocytes is recorded photographically suffers from the disadvantage that it is difficult to quantitate. Moreover, this technique may not be satisfactory when applied to study the migration of polymorphs towards soluble material (Hurley, 1963; Curran, 1967).

No quantitative *in vitro* technique for measuring the migration of polymorphonuclear-leucocytes (PMN) was available until Boyden (1962) developed his two compartment chamber technique. The technique involves the use of a chamber separated into two

compartments by a filter membrane, the pore size of which is such that PMN can pass through only by active migration. A suspension of PMN is placed on the upper side of the membrane and the test material on the lower. If the test material is chemotactic for PMN then the PMN will pass through the membrane. The chamber is incubated to allow time for polymorph migration. After incubation the membrane is removed, fixed and stained and the number of cells which have passed through the membrane is counted. The count obtained provides a measure of the effect of the test material on polymorph migration.

A quantitative <u>in vitro</u> measure of PMN migration was required which would enable a comparison of the chemotactic effect of different materials (some of them soluble) to be made.

Materials.

Oyster glycogen	: 20 mg/ml. in sterile saline.	
Medium	Gey's solution (Paul, 1961) containing 10 mg. streptomycin and 10,000 units penicillin/ml. and 20% inactivated normal rat serum.	
NaCl	8.00	gm/litre
KCl	0.375	"
$CaCl_2$	0.257	"
$MgCl_2 . 6H_2O$	0.210	"
$NaH_2PO_4 . 2H_2O$	0.150	"

KH_2PO_4	0.025	gm/litre
$NaHCO_3$	0.250	"

made up to 900 ml. with dionized water and autoclaved

100 ml. glucose solution (20 mg/ml.) sterilised by Sietz filtration

10 mg. streptomycin, and

10,000 units penicillin added

to 80 ml. of this solution 20 ml. heated (at 56°C. for ½ hour) normal rat serum was added.

Collection of Polymorphonuclear leucocytes

A rat was starved overnight and injected intraperitoneally with 10 ml. of a solution containing 20 mg. oyster glycogen/ml. in sterile isotonic saline. Three hours later the rat was killed with an overdose of ether. 15 ml. of medium was injected into the peritoneal cavity and the abdomen gently massaged. The abdominal skin was stripped from the muscle layers and a large incision made in the peritoneum. The exudate was collected with a sterile pipette. The exudate was not used if it was seen to be contaminated with erythrocytes. The cells were separated by centrifugation at 60-80 g for 3 minutes and resuspended in medium. The cells were counted and the cell concentration adjusted to about 5 c 10^6/ml.

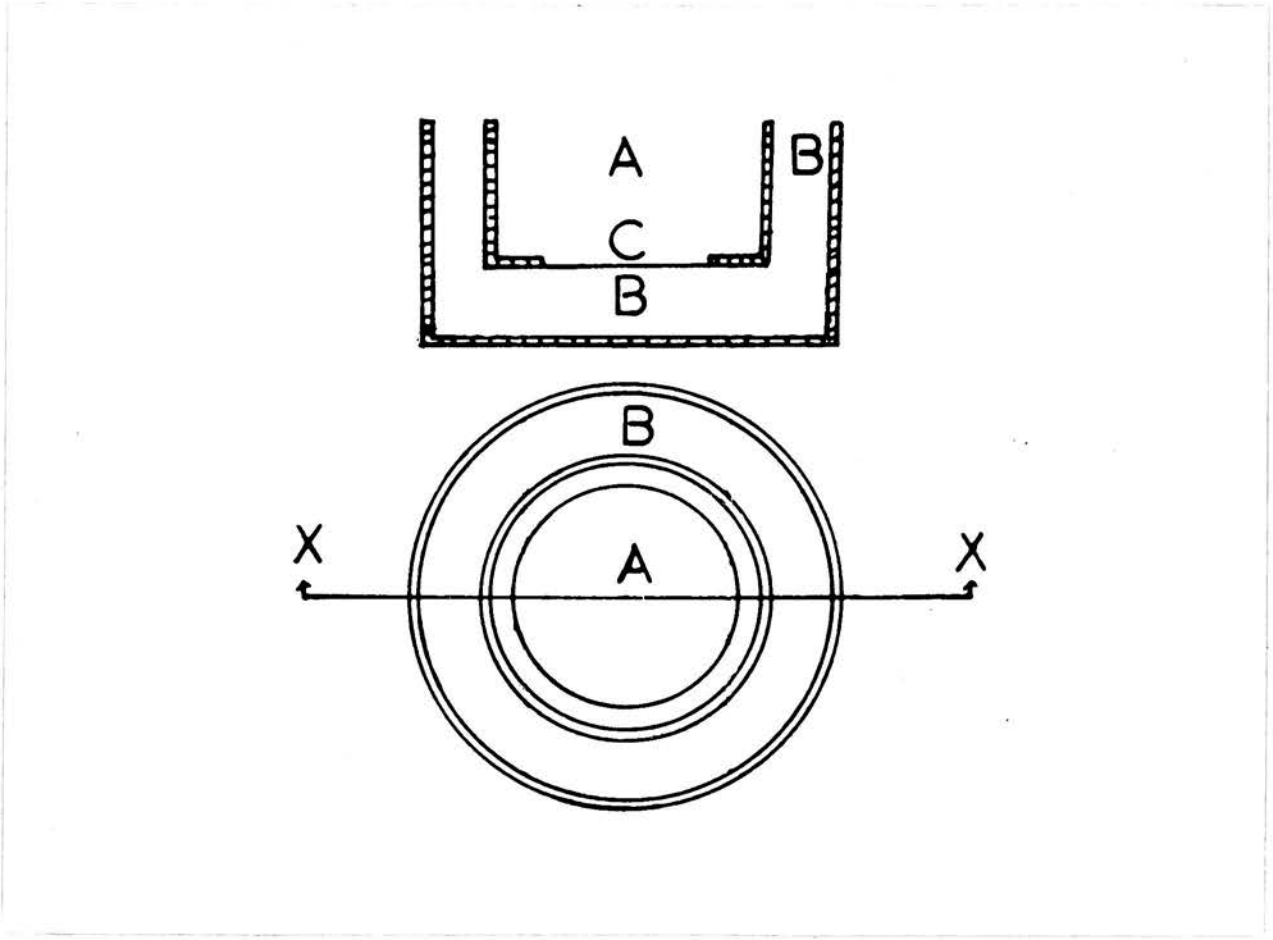
Procedure.

The modification of the two compartment chamber technique

(Boyden, 1962) described by Hurley (1963) was used. The apparatus consisted of two separate compartments. The upper compartment was a perspex cylinder and to its lower end a filter membrane of appropriate pore size, was glued. (UHU glue). The pore size of the filter was selected to minimise the number of PMN passing through the membrane by random migration whilst allowing a significant number of PMN to pass through the membrane by active migration. The optimal pore size was found to be 1.2 μ.

The lower compartment was a polypropylene tube of slightly larger diameter than the upper compartment. Both compartments were placed in a sterile chamber (under Ultra violet light) to keep bacterial contamination to a minimum. 1 ml. of the test material was added to the lower compartment using a sterile pipette and the upper compartment was then placed in position (see diagram). 1 ml. of the PMN suspension (about 5×10^6 cells) was added to the upper compartment with a sterile pipette. Each test was set up in triplicate. After incubation for 3 hours at 37°C. in air, the upper chamber was removed intact and the membrane fixed and stained by the following treatment:-

1. Fixed in methanol 5 seconds. Rinsed in distilled water.
2. Stained in Mayer's haematoxylin 5 minutes. Rinsed in distilled water.
3. 1% acid alcohol 1 minute. Rinsed in distilled water.
4. Blueing agent (saturated lithium carbonate) 2 minutes. Rinsed in distilled water.



Diagrammatic representation of chamber used to test materials for chemotactic activity.

A, compartment containing the PMN suspension.

B, compartment containing the test material.

C, membrane glued to under surface of A.

5. 70% ethanol 2 minutes.

6. 95% ethanol 2 minutes.

7. Absolute ethanol 3 minutes.

The glue used to cement the filter membrane to the upper compartment softened in ethanol and the membrane was removed with fine forceps. It was placed, with the lower surface facing upwards, on a microscope slide, allowed to dry and mounted in microscope immersion oil. The number of PMN migrating through the membrane was determined by counting the number of PMN on the surface of the membrane at a magnification of x 250. At least 10 fields were counted and the results were expressed as the number of PMN/field. The results from three tests were averaged.

Estimation of Protein by the Folin-Ciocalteu Method

This is a colourimetric method for the estimation of small (50-500 g) quantities of protein. The colour developed by the Folin-Ciocalteu phenol reagent in basic colution is due predominantly to its reaction with tyrosine and tryptophan residues of protein. The concentration of protein is directly proportional to the optical density of the coloured solution (Kabat & Mayer, 1961). The optical density of the coloured solution can be determined spectrophotometrically.

Materials.

Solution A: 2% sodium carbonate (5.4 gms. Na_2CO_3 . 10 H_20/100 ml. solution)

Solution B: 1% copper sulphate and 2% sodium tartrate, mixed 1:1 before using.

Solution C: 50 volumes of solution A mixed with 1 volume B immediately before using.

Folin & Ciocalteu's reagent (Hopkin & Williams, Chadwell Heath, Essex): diluted 1:1 with deionized water before use.

Procedure.

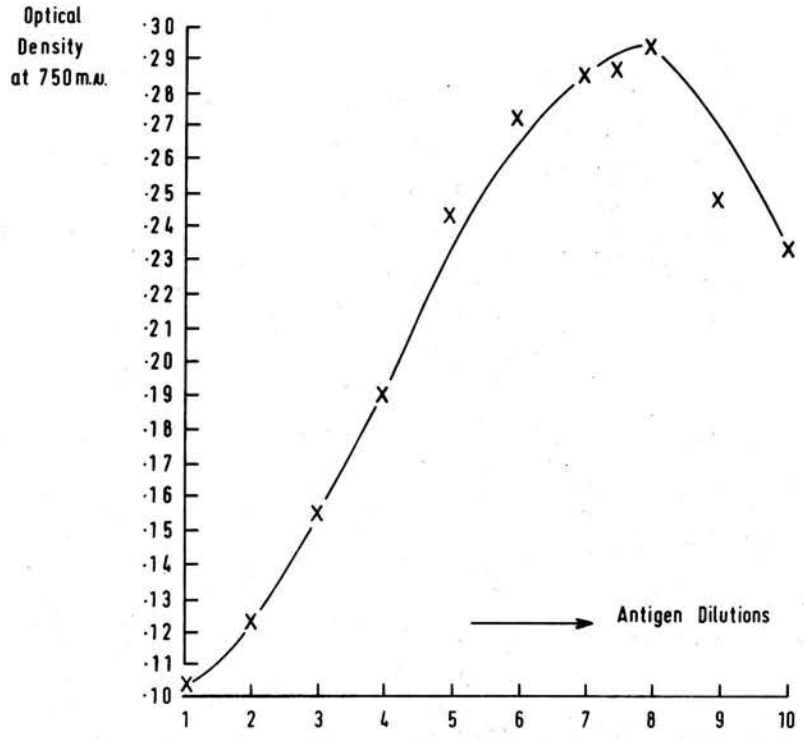
The method used was a modification of that described by Lowry, Rosebrough, Farr & Randall (1951). The technique was used to estimate the amount of protein precipitated by mixtures of antigen and antibody. To an immune precipitate in a tube was added 4.5 ml. solution C. The tubes were mixed immediately and allowed to stand at room temperature for 10 minutes. 0.5 ml. of the 1:1 Folin reagent was added and the mixture shaken vigorously. After standing at room temperature for 30 minutes in the dark the optical density of the solution was read at 750 mμ in a Unicam spectrophotometer.

Estimation of amounts of antigen and antiserum required to prepare an immune precipitate at equivalence

The equivalence concentration of an antiserum and the corresponding antigen were determined as follows. Constant amounts of the antiserum were mixed with varying quantities of the antigen

diluted in 0.85% saline in Wassermann tubes. The mixtures were allowed to stand at 37°C. for 1 hour and overnight at 4°C. The precipitate was spun down for 3×10^4 g. minutes and washed twice in saline. The protein content of the precipitate was measured as described above and the optical density of the solutions plotted against the antigen concentration as shown in the figure. From this graph the amounts of antigen and antiserum required to prepare an immune precipitate at equivalence were found.

Estimation of equivalence by quantitative precipitation and the Folin-Ciocalteu method.



RESULTS

RESULTS

Section (1) Incidence of a Factor reactive with tissue constituents in "normal" serum

The 4 drop complement fixation test, carried out in MRC pattern perspex plates with anti-complementary controls (see Materials and Methods p. 35), was employed to test sera for the presence of anti-tissue autoantibody. Over a period of two years "normal" adult albino rats, from the departmental animal house colony, were bled and their sera tested, without prior heating to inactivate complement, against whole liver homogenate (Materials & Methods p. 61).

Figure 1 shows that of 105 sera, 103 gave positive reactions with titres ranging from 1/8 to 1/512, 1 was negative and 1 was anticomplementary. It can be seen that the titres appear to follow a normal distribution.

Variation of titre with age

Preliminary investigation revealed that the sera of neonatal rats gave low titres in the complement fixation test with whole liver homogenate (see Table 1).

However, it was difficult to obtain sufficient sera to perform complement fixation tests, from rats during the first week of life, without considerably reducing their chances of survival. Accordingly, rats were bled weekly from one week after birth and their sera collected and stored at -25°C. until required for testing. In order to reduce any variation in titre attributable to changes in the conditions of testing, the sera were tested

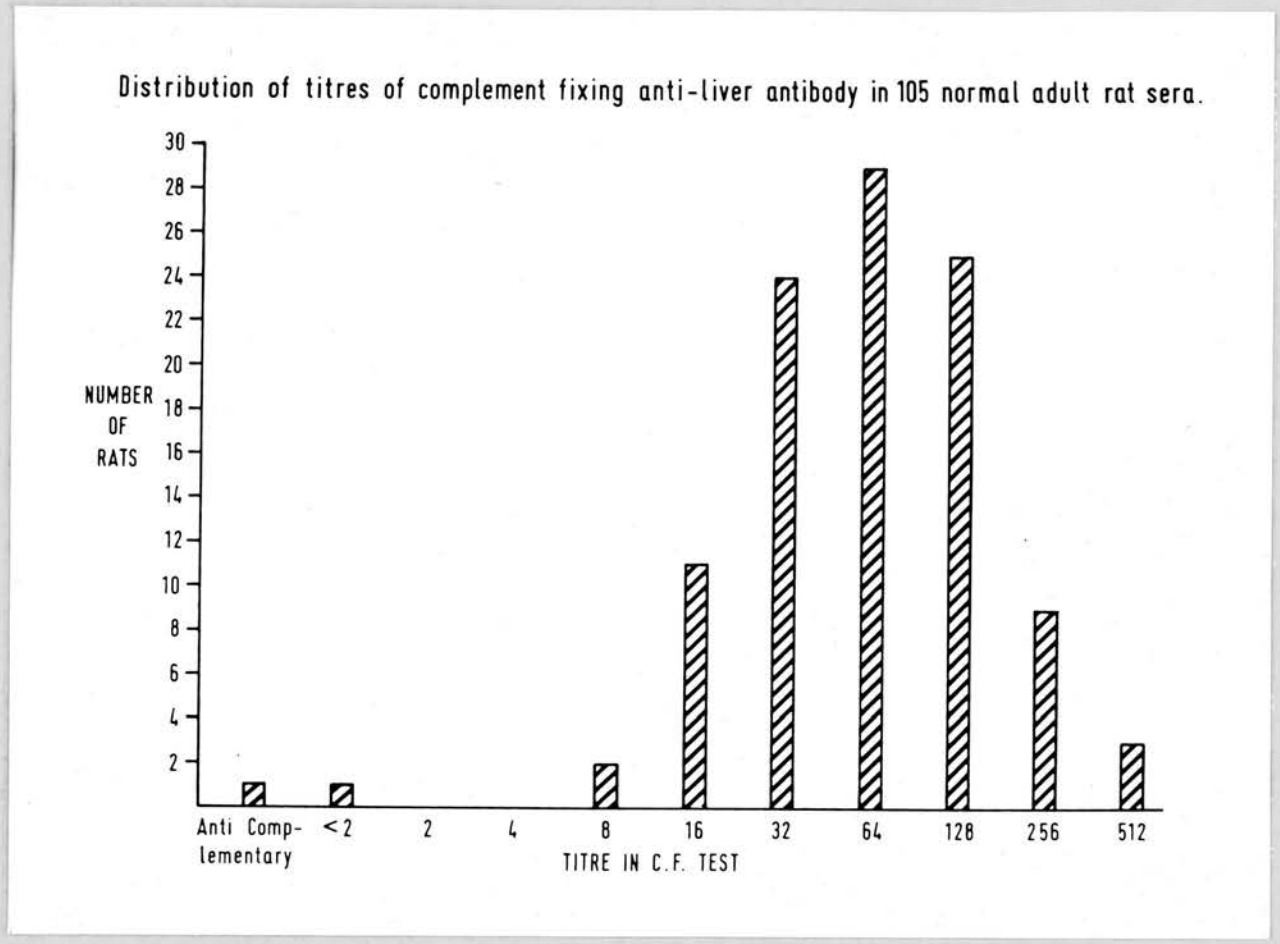

Distribution of titres of complement fixing anti-liver antibody in 105 normal adult rat sera.
NUMBER OF RATS
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
Anti Comp-lementary
<2
2
4
8
16
32
64
128
256
512
TITRE IN C.F. TEST

Figure 1

Table 1

Complement Fixation titres against whole liver homogenate of rat sera taken at intervals after birth

Age	Number of rats with titre of								Mean titre
	2	2	4	8	16	32	64	128	
24 hours	2	1							
3 days	1		2	3					
5 days	1		1		2				
1 week	2		2						
2 weeks	1			1	5	1			13
3 weeks					1	3	3	1	49
4 weeks					1	3	2	2	52

simultaneously. The results (Figure 2) show that the complement fixation titres rose rapidly over the first 3 weeks of life reaching adult levels by the time the animal was 3-6 weeks old.

Reaction with Autologous Material

It appeared necessary to determine whether the serum factor would react with autologous material. Four rats were bled and their livers removed and homogenised. The sera were tested in the complement fixation test against autologous and homologous liver homogenates and Table 2 shows that the serum factor is able to react with autologous material.

Table 2

Titres in Complement Fixation test against autologous and homologous liver homogenate.

Rat	Titres	
	Autologous Liver homogenate	Homologous Liver homogenate
1	32	32
2	128	64
3	128	64
4	32	32

Activity against various rat organ and tissue homogenates

Eleven rat sera were tested against several rat organ and tissue homogenates. The results (Table 3) showed that there

Complement fixation titres of sera taken at intervals after birth of 12 normal rats. The line is drawn through the mean titre at each interval.

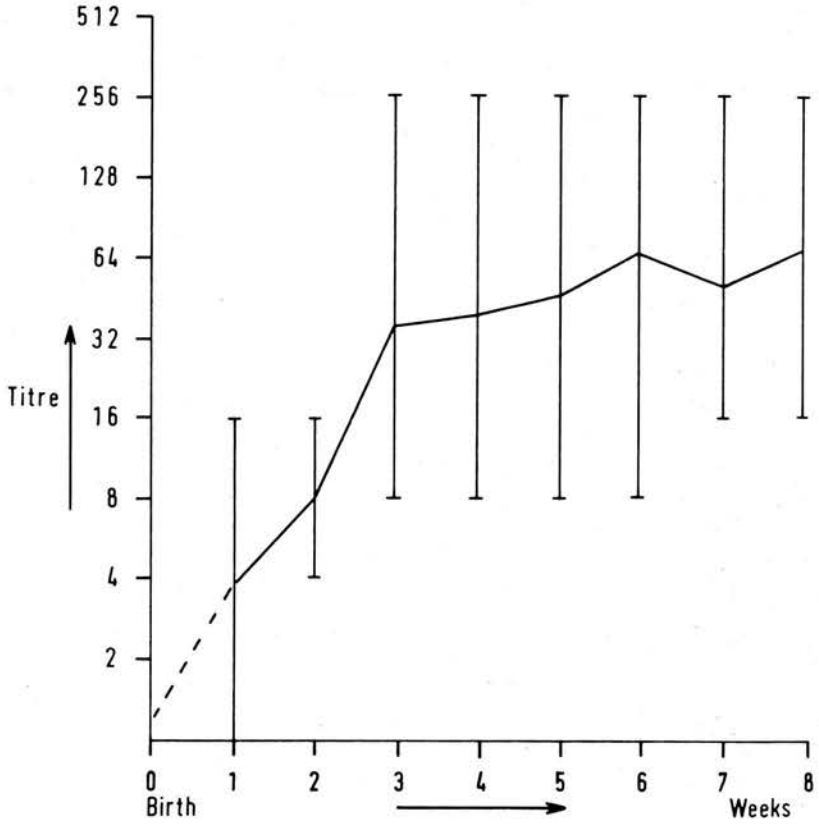


Figure 2

was some variation in titre against the various homogenates in different animals, for example, compare rat 1:- 1/128 against liver and 1/256 against thymus, with rat 5:- 1/64 against liver and 1/16 against thymus. This raises the possibility that, to a small extent, the factor might be tissue specific. On average the highest titres recorded were against kidney and the lowest titres against heart and muscle.

Comparison of activity as measured by complement fixation and passive haemagglutination

If the reaction described above, between normal rat serum and tissue constituents, was a result of antigen-antibody combination then it seemed likely that this reaction should be detectable by other antibody assays. Eight "normal" unheated rat sera were tested against various rat organ and tissue homogenates in the complement fixation and passive haemagglutination* tests. The results are shown in Table 4. It is evident that, although there is no close correlation between individual titres obtained by the two tests, in many instances (e.g. serum 4) high titres in the complement fixation test are associated with high titres in the passive haemagglutination test.

Activity of "normal" serum from various species against homologous tissues

The finding of a serum factor reactive with tissue homogenates in rat sera, together with the report of a similar factor in rabbit sera (Kidd & Friedewald, 1942 a,b) suggested that the

* The passive haemagglutination tests were kindly performed by Mrs. D.E. Suckling.

Table 3

Complement Fixation Titres of Unheated Rat Sera Tested against Rat Organ and Tissue Homogenates

Serum No.	Liver	Spleen	Thymus	Heart	Muscle	Kidney	Lung	Brain
1	128	128	256	128	256	128	64	16
2	32	128	128	-	32	128	64	256
3	128	64	64	32	16	128	64	32
4	64	128	64	128	16	128	32	128
5	64	64	16	-	-	32	32	128
6	64	32	16	16	-	128	32	64
7	128	128	128	64	32	512	32	64
8	128	128	128	64	32	256	64	128
9	128	64	32	128	-	256	64	128
10	256	256	256	128	64	512	128	128
11	256	256	128	128	64	256	128	256

- = less than 1/4.

Table 4

Comparison of Activity in Complement Fixation (C.F.) and Passive Haemagglutination (P.H.) tests against various rat organ and tissue homogenates

Serum	Liver		Spleen		Thymus		Heart		Muscle		Kidney		Lung		Brain	
	C.F.	P.H.	C.F.	P.H.	C.F.	P.H.	C.F.	P.H.	C.F.	P.H.	C.F.	P.H.	C.F.	P.H.	C.F.	P.H.
1	128	4	64	4	32	4	128	8	0	4	256	4	64	4	128	-
2	128	4	128	4	128	4	64	8	32	4	512	8	32	4	64	-
3	128	16	128	16	128	8	64	4	32	8	256	16	64	8	128	-
4	256	64	256	32	256	128	128	64	64	32	512	64	128	64	128	-
5	A.C.	16	A.C.	16	A.C.	16	A.C.	16	A.C.	16	A.C.	16	A.C.	16	A.C.	-
6	256	8	256	8	128	16	128	16	64	8	256	16	128	8	256	-
7	32	8	32	8	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	512	16	512	8	256	16
8	32	4	N.T.	0	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	256	4	512	4	64	0

Results expressed as reciprocal of titre

A.C. = anticomplementary serum controls

\- = controls not stabilised

0 = less than 1/4.

N.T. = not tested

serum of "normal" animals from other species may contain a factor reactive with autologous tissue constituents. A small survey of the anti-tissue complement fixing activity of sera from animals of other species was carried out.

In order to test unheated "normal" guinea pig sera in the complement fixation test it was first necessary to remove complement. This was achieved by treating the sera with a complex of bovine serum albumin - rat antibovine serum albumin. These sera were then tested in the complement fixation test against guinea pig liver homogenate. No complement fixing activity was detected in the six sera tested.

Six hamster sera were tested in the complement fixation test against homologous liver homogenate. Four gave titres of 1/128 and two titres of 1/64. Three hamster sera were tested against autologous liver homogenate; two gave titres of 1/64 and one a titre of 1/32. Two sera were tested before and after heating at 56°C. for 30 minutes against homologous liver homogenate. The titre of one sera was reduced by heating from 1/128 to 1/16 and of the other from 1/16 to $<$1/2.

The sera from 10 outbred mice were taken and tested in the complement fixation test against homologous liver homogenate. All the sera were found to be anti-complimentary.

Section (2) Heat Stability of serum factor and the response to tissue breakdown

Weir (1963) noted the presence of complement fixing anti-liver antibody in some 15 per cent of heated (56°C. for 30 minutes) "normal" rat sera. A similar incidence was noted in this work in which 15 of the 56 heated (56°C. for 30 minutes) "normal" rat sera tested were found to be positive in the complement fixation test against homologous liver homogenate. Table 5 shows the complement fixing activity of eight of these sera, before and after heating at 56°C. for 30 minutes, against various rat organ and tissue homogenates. It can be seen that the activity was reduced or removed by heating at 56°C. before testing. The activity against kidney appears to be least affected.

Table 6 shows the effect of temperature on the activity of a "normal" rat serum pool in the complement fixation test against rat liver and kidney homogenates.

Table 6

Tested against	Titres in Complement Fixation test after standing at			
	-25°C.	4°C. for 16 hrs.	room temperature for 3 hours	room temperature for 6 hours
Liver homogenate	128	128	128	64
Kidney homogenate	128	128	64	64

Table 5

Complement Fixation titres of "normal" rat sera, before and after heating at 56°C. for 30 minutes against various organ and tissue homogenates

Sera No.		Liver	Heart	Kidney	Brain	Muscle	Spleen	Lung
1	U	256	64	256	512	32	512	512
	H	0	0	64	4	0	0	0
2	U	256	256	128	512	8	128	512
		16	0	16	0	0	32	512
3	U	128	64	64	512	8	64	128
	H	4	0	0	4	0	0	4
4	U	64	16	16	64	16	32	128
	H	8	0	16	0	0	4	16
5	U	256	8	32	256	0	256	128
	H	0	0	0	0	0	0	0
6	U	32	8	16	512	0	16	32
	H	0	0	0	4	0	16	0
7	U	32	0	32	16	0	32	32
	H	0	0	0	0	0	0	0
8	U	128	32	64	64	32	128	128
	H	0	0	16	0	0	0	0

U = unheated sera

H = sera heated at 56°C. for 30 minutes.

0 = <4

One aliquot was placed directly in the deep freeze and others were allowed to stand at 4°C. for 16 hours and room temperature for 3 and 6 hours. The aliquots were tested simultaneously and it can be seen that standing at 4°C. for 16 hours does not affect the titre. However a distinct reduction in titre took place after the serum pool had stood at room temperature for 6 hours.

Response to tissue breakdown

The induction of tissue damage by the injection of carbon tetrachloride (CCl_4) into rats has been found to lead to the appearance of a heat stable anti-liver autoantibody in their sera (Weir, 1963). It seemed necessary, therefore, to determine whether or not the production of the heat labile serum factor, described in this work, could similarly be stimulated by tissue breakdown.

Rats were injected subcutaneously with 0.03 ml. CCl_4/100 gm body weight (a dose known to cause liver damage in rats, Cameron & Karunaratne, 1936; Weir, 1963). They were bled at intervals and their sera collected and stored at -25°C. until required for testing. The activity of these sera, before and after heating at 56°C. for 30 minutes, was assayed in the complement fixation test against homologous liver homogenate. The results are shown in figures 3 and 4, and the data from which these figures were compiled are recorded in table 7. It is evident that there is

Complement fixation titres with sera from rats & rat liver antigen at intervals after CCl_4. The curve is drawn through the average titre at each interval

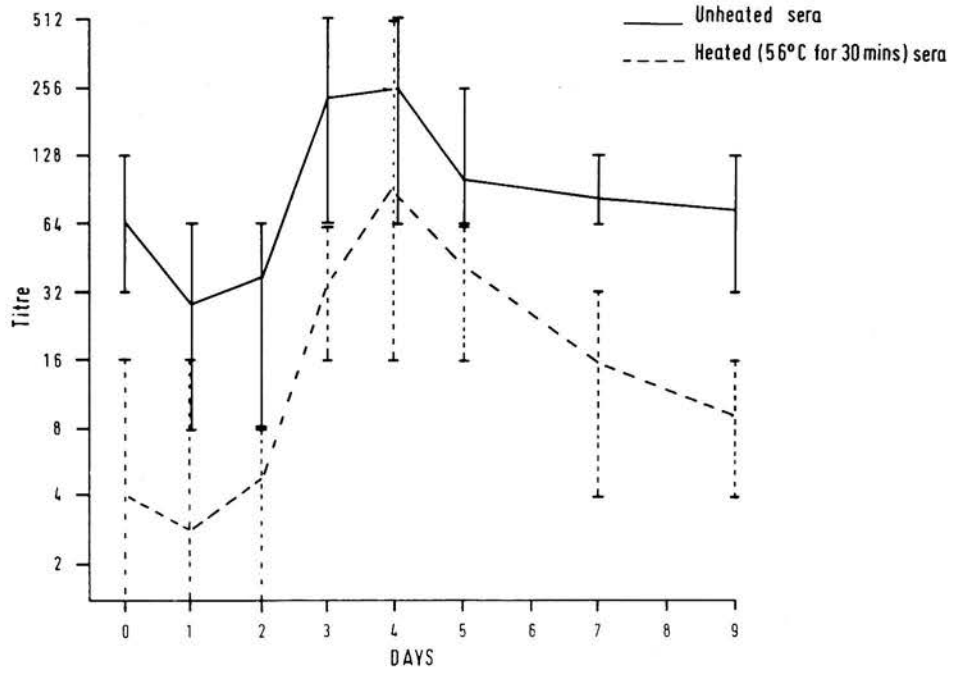


Figure 3

Complement fixation titres of heat labile anti-liver antibody at intervals after CCl_4 (obtained by subtraction of heat stable titre from the titre of unheated sera). The curve is drawn through the average titre at each interval.

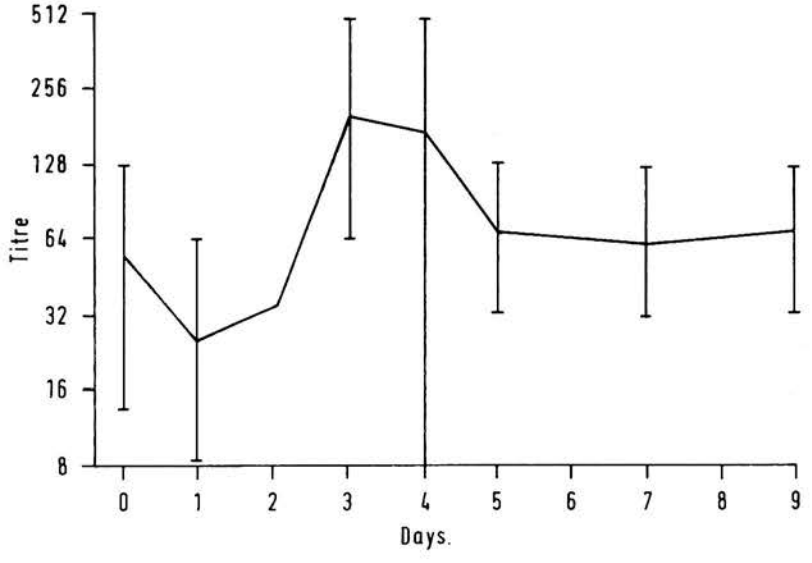


Figure 4

Table 7

Complement Fixation test against homologous liver homogenate
Titres of sera before and after heating at 56°C. for 30 mins
from rats injected with carbon tetrachloride

Unheated

Animal	0	1	2	3	4	5	7	9days
1	128	32	64	128	256	256	64	
2	32	32	16	256	512		64	
3	64	32	64	256	256	128	64	
4	128	64	64		512	64		
5	64	16	16		128	64		
6	64	16	64		128			
7	16	8	32	512	128	64	128	32
8	32	32	8	64	64			
9	64	32			256	64	128	128
No. of Animals	9	9	8	5	9	7	5	2
Mean Titre	66	29	49	243	249	110	90	80

Heated

Animal	0	1	2	3	4	5	7	9days
1	16	8	8	64	128	64	32	
2	4	16	8	32	512		32	
3	4	<2	<2	32	32	32	8	
4	<2	<2	4		64	64		
5	<2	<2	8		32	64		
6	<2	<2	8		32	64		
7	4	<2	4	16	32	8	4	4
8	4	<2	<2	32	32			
9	<2	4			16	8	4	16
No. of Animals	9	9	8	5	9	7	5	2
Mean Titre	4	3	5	35	98	43	16	10

Unheated mean titre - Heated Mean titre

57	26	44	208	151	67	64	70

a marked increase in titre of anti-liver serum factors following the induction of liver damage by CCl_4.

A further attempt to study the effect of tissue damage on the production of the heat labile anti-tissue serum factor was made by injecting anti-~~nephritic~~ kidney serum into rats. An anti-nephritic serum was prepared in rabbits. It was heated at 56°C. for 30 minutes to inactivate complement and absorbed with washed rat erythrocytes and rat serum. It was tested by passive haem-agglutination and found to give a high titre against rat kidney homogenate. One ml. of this anti-nephritic serum was injected intravenously into each of 5 rats. The rats were bled at intervals and their serum tested against homologous kidney homogenate in the complement fixation test. Unfortunately only two rats survived this treatment. The results are recorded in Table 8.

Table 8

Titres in complement fixation test against rat kidney homogenate of sera taken at intervals after the injection of anti-nephritic serum.

		Titres in Complement Fixation Test					
		0	2	4	6	9	12 days
rat 1	U	64	128	256	128	32	
	H	8	8	32	<2	8	
2	U	64	128	128	32	128	128
	H	4	<2	<2	16	<2	2

U = Unheated sera

H = Sera heated at 56°C. for 30 mins.

Section (3) Physico - Chemical Properties of the anti-tissue serum factor

The finding of a heat labile anti-tissue factor, detectable by complement fixation and passive haemagglutination and apparently stimulated by tissue breakdown products, in "normal" rat serum raised the question as to whether this factor was an autoantibody. To clarify this problem an attempt was made to define the physico-chemical nature of the factor and it was hoped that this knowledge would, in turn, shed some light on the biological activity of the factor.

Column Chromatography on Sephadex G. 200

Six "normal" rat sera and two rat sera taken 4 days after the injection of CCl_4, were fractionated by chromatography on Sephadex G200 (Materials & Methods p. 53). The protein distribution pattern obtained is shown in Figure 5. Fractions were taken from the ascending portions of the four peaks and the descending portion of the first. The fractions were tested against various rat organ and tissue homogenates in complement fixation test. The results are tabulated in Table 9 and it can be seen that any activity remaining after fractionation is localised in fraction 1 and 2. The anti-kidney activity appears to be most consistently recoverable. A number of high titred sera were fractionated by Sephadex chromatography and the fractions tested against rat kidney homogenate in the complement fixation test. These results are shown

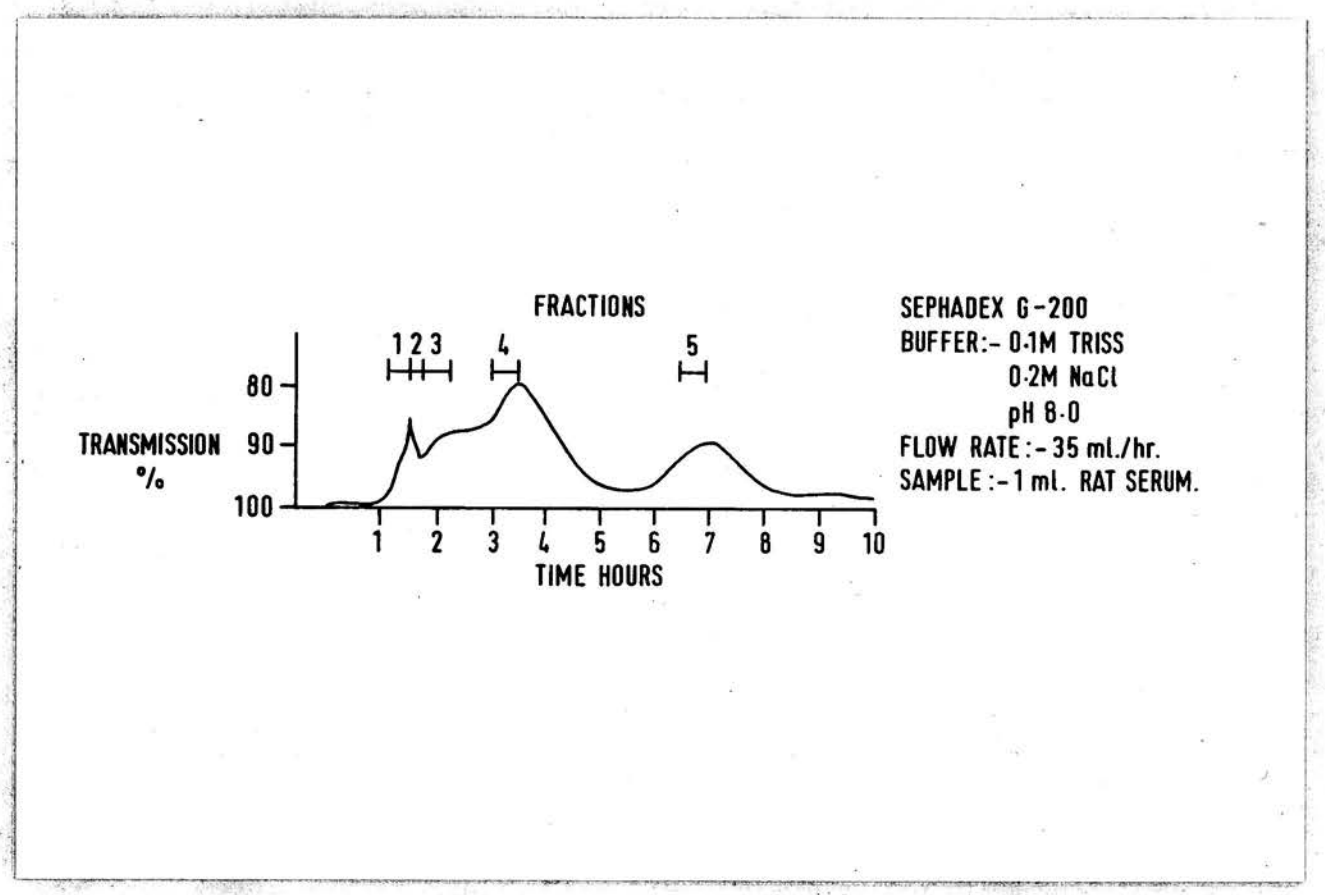


Figure 5

Sephadex G200 separation of rat serum indicating fractions taken

Table 9

Titres in complement fixation test against various rat organ and tissue homogenates of sera and serum fractions obtained by chromatography on Sephadex G200

Serum or Fraction	Liver	Spleen	Thymus	Heart	Muscle	Kidney	Lung	Brain
1	128	128	256	128	256	128	64	16
F1	-	-	-	-	8	-	-	-
F2	-	-	8	-	16	8	-	-
F3	-	-	-	-	-	-	-	-
F4	-	-	-	-	-	-	-	-
F5	-	-	-	-	-	-	-	-
2	32	128	128	0	32	128	64	256
F1	-	-	-	-	-	-	-	-
F2	-	-	-	-	-	-	-	-
F3	-	-	-	-	-	-	-	-
F4	-	-	-	-	-	-	-	-
F5	-	-	-	-	-	-	-	-
3	128	64	64	32	16	128	64	32
F1	8	-	-	-	-	16	-	-
F2	-	-	-	-	-	-	-	-
F3	-	-	-	-	-	-	-	-
F4	-	-	-	-	-	-	-	-
F5	-	-	-	-	-	-	-	-
4	8	-	-	-	-	16	-	4
F1	-	-	-	-	-	-	-	-
F2	-	-	-	-	-	8	-	-
F3	-	-	-	-	-	-	-	-
F4	-	-	-	-	-	-	-	-
F5	-	-	-	-	-	-	-	-
5	64	128	64	128	16	128	32	128
F1	-	-	-	-	-	-	-	-
F2	-	-	-	-	-	8	-	16
F3	-	-	-	-	-	-	-	-
F4	-	-	-	-	-	-	-	-
F5	-	-	-	-	-	-	-	-

- = <4

Table 9

Serum or Fraction	Liver	Spleen	Thymus	Heart	Muscle	Kidney	Lung	Brain
6	64	64	16	0	0	32	32	128
F1	-	-	-	-	-	8	-	8
F2	-	-	-	-	-	-	-	8
F3	-	-	-	-	-	-	-	-
F4	-	-	-	-	-	-	-	-
F5	-	-	-	-	-	-	-	-
C1 (D4 CC14)	128	128	128	64	32	128	64	128
F1	8	-	-	-	-	-	-	-
F2	32	8	-	-	-	16	8	8
F3	-	-	-	-	-	-	-	-
F4	-	-	-	-	-	-	-	-
F5	-	-	-	-	-	-	-	-
C2 (D4 CC14)	512	256	128	128	-	256	128	64
F1	128	8	-	-	-	32	16	-
F2	16	-	-	-	-	8	-	-
F3	-	-	-	-	-	-	-	-
F4	-	-	-	-	-	-	-	-
F5	-	-	-	-	-	-	-	-

- = <4

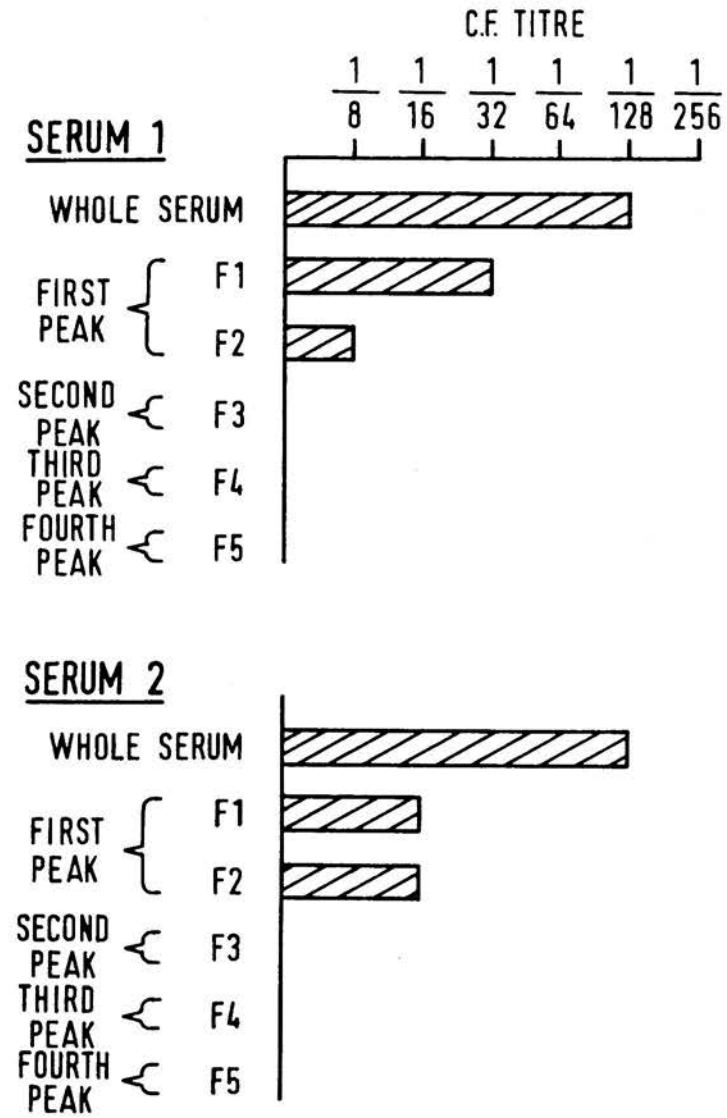


Figure 6

Complement fixation titres of rat serum fractions (Fig. 5) tested against rat kidney homogenate

in Figure 6. An attempt was made to concentrate the activity by dialysis of fraction 1 and 2 against carboxy-methyl cellulose. All activity was lost by this procedure.

The content of the fractions was checked by immunoelectrophoretic analysis. Each fraction was electrophoresed and precipitated by rabbit anti-rat sera. Fractions 1 and 2 contained the IgM and α_2 macroglobulins and were free from contaminating IgG. Fraction 4 contained IgG and fraction 5 albumin.

Zone centrifugation on sucrose density gradients

Eight "normal" rat sera and two rat sera taken 4 days after the injection of CCl_4 were fractionated by zone centrifugation on sucrose density gradients (Materials & Methods p.55). Nine fractions were taken (see Fig. 7) and tested in the complement fixation test against various rat organ and tissue homogenates. The results are recorded in Table 10 and it can be seen that the complement fixing activity is localised mainly in fractions 6 and 7. A large proportion of the activity was recovered against all the organ and tissue homogenates in contrast to the poor recovery obtained by Sephadex chromatography (c.F. sera 2-6 in Tables 11 and 12). Some of the fractions were tested by passive haemagglutination (by Mrs. D.E. Suckling) and the activity was similarly localised in fractions 6 and 7.

The content of the fractions was checked by immunoelectrophoretic analysis. Fractions 3 and 4 contained IgG and fractions

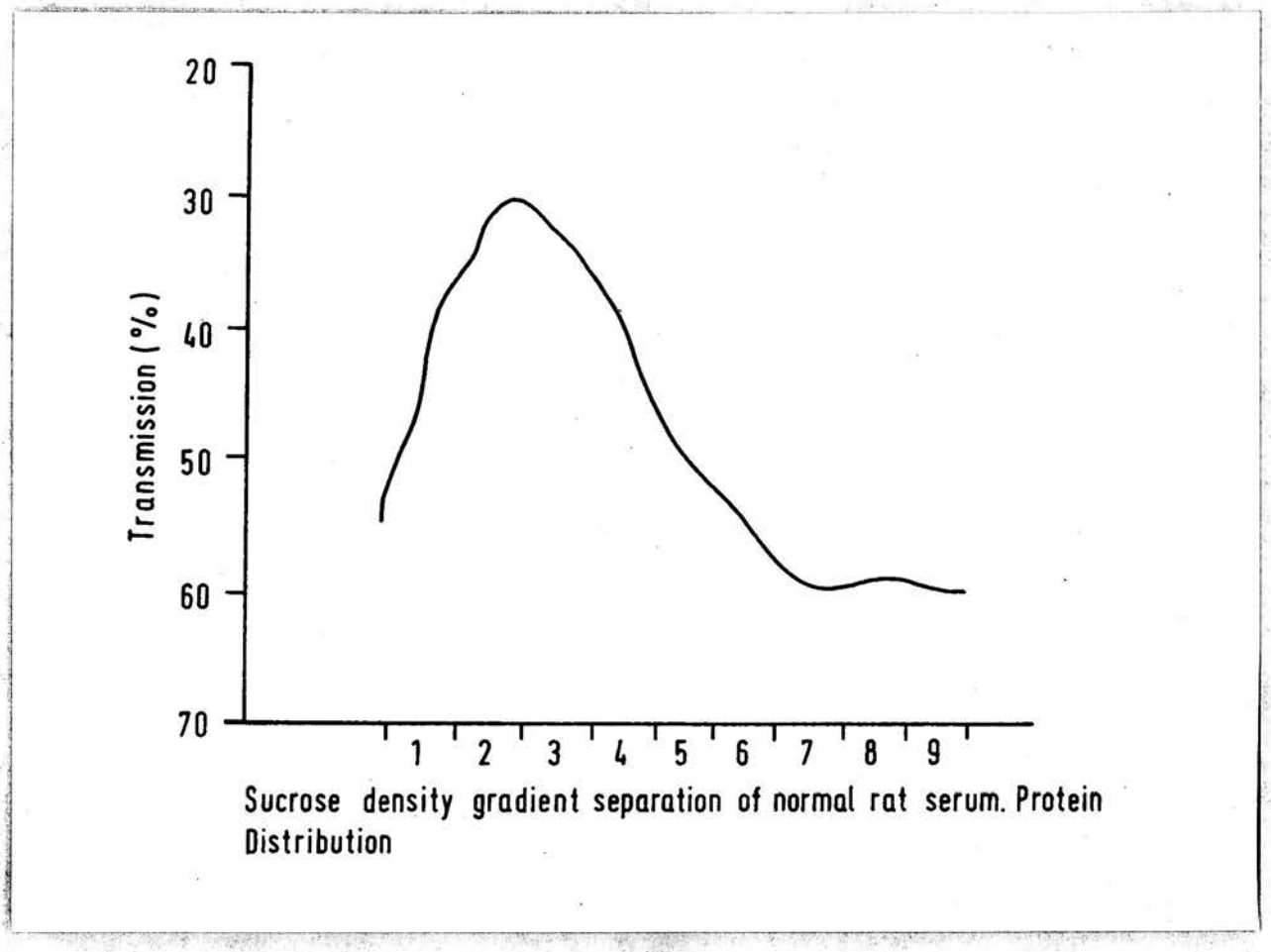


Figure 7

Sucrose density gradient separation of normal rat serum indicating fractions taken

Table 10

Titres in Complement Fixation against various rat organ and tissue homogenates of sera and serum fractions prepared by zone ultracentrifugation

Serum or Liver Fraction		Spleen	Thymus	Heart	Muscle	Kidney	Lung	Brain
2	32	128	128	-	32	128	64	256
F_{1-4}	-	-	-	-	-	-	-	-
F_5	-	-	-	-	-	-	-	-
F_6	8	16	16	-	8	32	8	32
F_7	4	4	4	-	8	16	8	16
F_8	-	-	-	-	-	-	-	-
F_9	-	-	-	-	-	-	-	-
3	128	64	64	32	16	128	64	32
F_{1-4}	-	-	-	-	-	-	-	-
F_5	-	-	-	-	-	8	-	-
F_6	4	4	4	8	8	64	16	32
F_7	4	4	-	4	-	32	8	16
F_8	-	-	-	-	-	8	-	-
F_9	-	-	4	4	-	16	-	8
4	16	16	8	4	-	64	8	32
F_{1-4}	-	-	-	-	-	-	-	-
F_5	-	-	-	-	-	-	-	-
F_6	8	4	4	4	-	8	-	8
F_7	8	4	-	4	-	16	4	16
F_8	-	-	-	-	-	8	4	16
F_9	-	-	-	-	-	4	-	8

Serum or Fraction	Liver	Spleen	Thymus	Heart	Muscle	Kidney	Lung	Brain
5	64	32	32	16	4	128	32	128
F_{1-4}	-	-	-	-	-	-	-	-
F_5	-	-	-	-	-	-	-	-
F_6	-	-	-	-	-	-	-	-
F_7	8	4	4	4	-	16	4	8
F_8	8	8	4	4	-	16	4	16
F_9	8	-	-	-	-	8	-	8
6	64	64	16	-	-	64	32	128
F_{1-4}	-	-	-	-	-	-	-	-
F_5	-	-	-	-	-	-	-	-
F_6	4	8	8	-	-	8	-	-
F_7	-	-	-	-	-	16	8	16
F_8	-	-	-	-	-	8	4	8
F_9	-	-	-	-	-	-	-	-
7	64	32	16	16	-	128	32	64
F_{1-4}	-	-	-	-	-	-	-	-
F_5	-	-	-	-	-	-	-	-
F_6	8	4	4	4	-	16	4	16
F_7	8	4	4	4	-	8	-	8
F_8	-	-	-	-	-	-	-	-
F_9	-	-	-	-	-	-	-	-
8	16	16	8	8	-	64	4	64
F_{1-4}	-	-	-	-	-	-	-	-
F_5	-	-	-	-	-	-	-	-
F_6	-	-	-	-	-	-	-	-
F_7	16	8	16	8	4	16	8	8
F_8	-	4	4	4	-	16	4	16
F_9	-	-	-	-	-	4	-	-

Serum or Fraction	Liver	Spleen	Thymus	Heart	Muscle	Kidney	Lung	Brain
$C_1(CCl_4D_4)$	256	64	64	64	-	256	64	128
F_{1-4}	-	-	-	-	-	-	-	-
F_5	-	-	-	-	-	-	-	-
F_6	32	8	8	4	-	16	-	16
F_7	8	4	4	-	-	4	4	8
F_8	16	8	8	4	-	16	8	16
F_9	4	-	-	-	-	4	-	4
$C_2(CCl_4D_4)$	128	128	128	64	32	256	64	64
F_{1-4}	-	-	-	-	-	-	-	-
F_5	-	-	-	-	-	-	-	-
F_6	16	8	8	8	4	32	8	16
F_7	16	8	8	8	8	8	8	16
F_8	4	4	-	-	-	4	-	4
F_9	4	-	-	-	-	8	4	-

- = <4

6 and 7 IgM with no detectable contaminating IgG.

Preparative electrophoresis

Two "normal" rat sera were fractionated by horizontal zone electrophoresis in a Pevikon supporting medium at 4°C. Fourteen fractions were obtained and tested in the complement fixation test against rat liver homogenate. No activity could be detected as all fractions were anti-complementary.

These results show that the complement fixing activity against rat organ and tissue homogenates in "normal" rat sera is eluted with the IgM and α_2 macroglobulin from Sephadex G200 and is associated with the high molecular weight serum components sedimenting with the IgM on sucrose density gradient fractionation. The activity detectable by passive haemagglutination behaves similarly on fractionation by zone ultracentrifugation. These results are in accord with the heat labile serum factor being an immunoglobulin M and confirm that the auto-antibody induced by tissue damage is an IgM.

Section (4) Antigens reactive with anti-tissue serum factor

The complement fixing autoantibody induced in rats by toxic liver injury has been found to be directed against the sedimentable components of rat liver homogenate (Weir, 1963; Elson, 1965; Pinckard & Weir, 1966). It seemed important to ascertain whether or not the heat labile immunoglobulin M, described in this work, was directed against similar antigens. It also seemed possible that the characterisation of these antigens might help to elucidate the apparent failure of animals to develop tolerance to these tissue antigens.

An attempt was made to solve this problem by comparing the antigen content of sub-cellular fractions prepared by differential centrifugation of tissue homogenates (Materials & Methods p. 62). This was achieved by performing quantitative complement fixation tests using constant amounts of anti-sera and varying dilutions of sub-cellular fractions. The various fractions, each made up to correspond to their concentration in the whole tissue homogenate, were diluted in doubling dilution series. 1.25 MHD guinea pig complement and an appropriate dilution of a standard serum pool were added. From this point the method described previously (Materials & Methods p.38) was followed and the end point taken as the last tube in the antigen dilution series which showed clearly visible sheep erythrocytes.

The results of tests with a normal rat serum pool and liver

fractions are shown in Fig. 8. It can be seen that the liver antigen which fixes complement with normal rat serum is primarily associated with the "mitochondrial fractions" of rat liver with the main peak of activity in fraction 3 (F_3). No activity was found in fraction 7 (F_7), the fraction containing the "cell sap" or soluble antigens. Further tests (Fig. 9 and 10) with normal serum and serum taken 4 days after the injection of CCl_4 and fractions obtained from kidney homogenate revealed a similar distribution of antigenic activity.

Cross absorption Studies

The results shown in Table 3 indicated that there was some variation of the complement fixing activity against the various organ and tissue homogenates in the sera of different animals. This suggested that the heat labile anti-tissue IgM may be directed against some tissue or organ specific antigens. The purpose of the following cross absorption studies was to determine whether this was so.

At least 2.0 ml. of each rat serum was required for cross absorption studies. Eight 0.2 ml. aliquots were absorbed with one of eight rat tissue or organ preparations and the remainder of the serum was stored at -25°C. The eight preparations tested were:- liver, spleen, heart, muscle, kidney, lung and brain homogenates and a mitochondrial preparation of liver (F_3). The 0.2 ml. aliquots were absorbed with 0.6 ml. of the washed (three

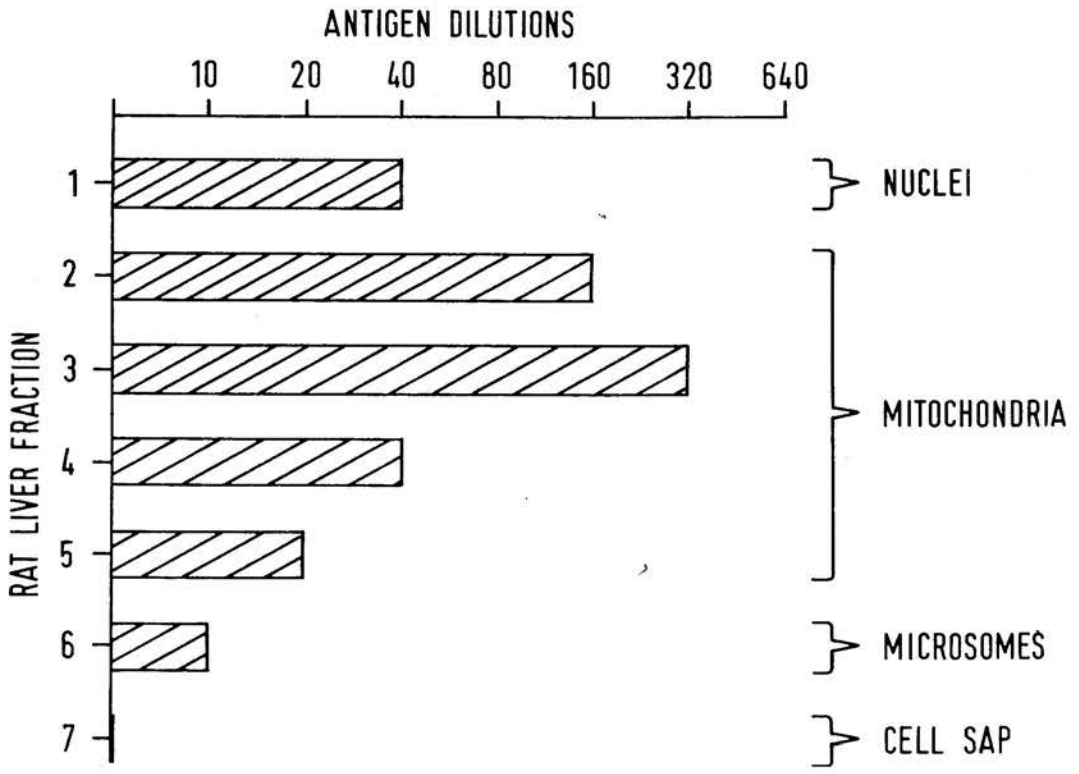
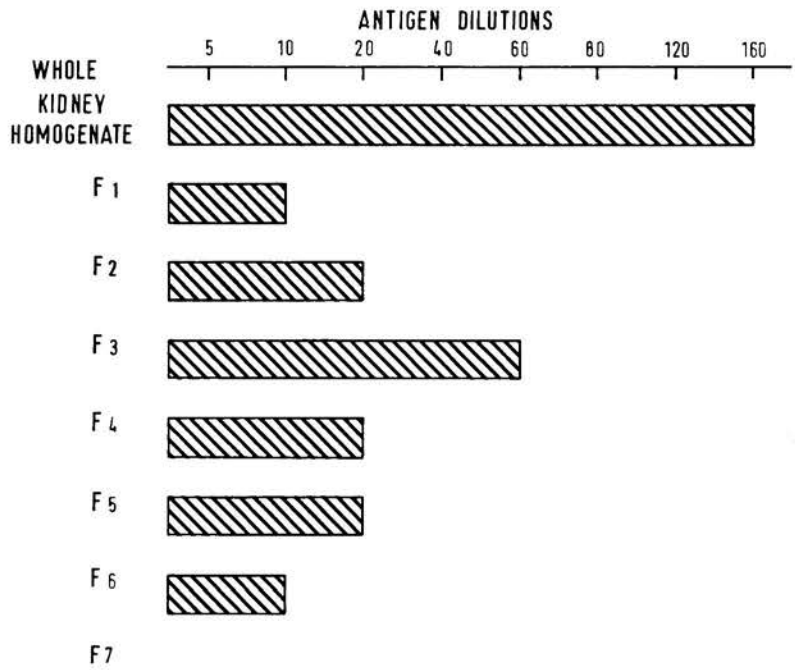


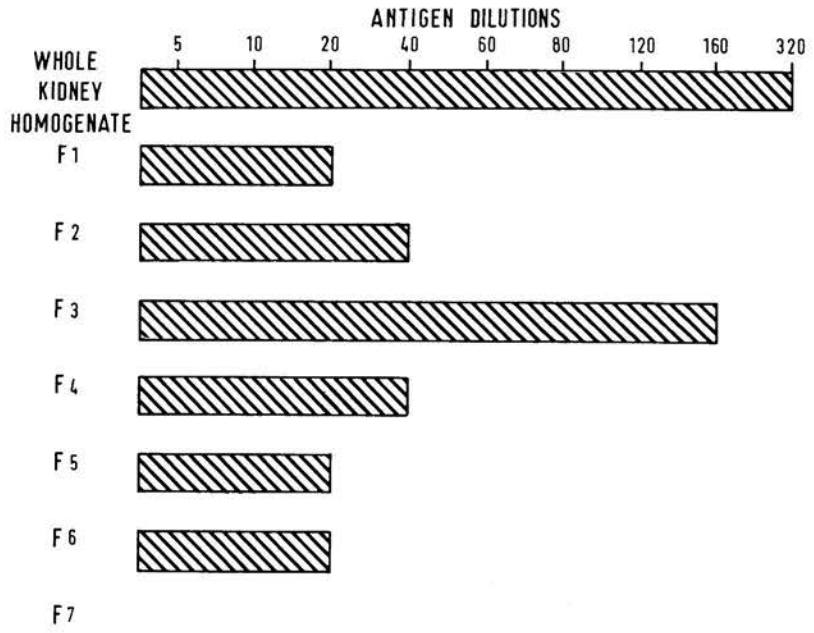
Figure 8

Antigen dilution test with rat serum pool and liver fractions.
Blocks represent positive complement fixation test
at respective antigen dilution.



Antigen dilution test with standard serum & kidney fractions. Blocks represent positive complement fixation test at respective antigen dilutions

Figure 9



Antigen dilution test with standard serum (4 days after CCl_4) & kidney fractions. Blocks represent positive complement fixation test at respective antigen dilutions.

Figure 10

times in 0.25 M sucrose and spun down by centrifugation for 6×10^6 g min.) preparation. The mixture was allowed to stand at room temperature for 20 minutes and 2 hours at 4°C. It was then centrifuged for 6×10^6 g minutes and the supernate removed. The supernate was reabsorbed with 0.6 ml. of the appropriate preparation as before and re-centrifuged. Complement fixation tests were performed on the original sera and on each absorbed aliquot, against all the preparations used. 12 "normal" rat sera and 4 sera taken 4 days after the injection of CCl_4, were tested.

The results showed that the absorption of a serum with a particular homogenate removed the activity against all the other tissue and organ preparations tested. Some activity remained in sera absorbed with F_3 and occasionally some activity remained in sera absorbed with muscle. However, the activity against the preparations was found to be reduced or removed in control sera mixed with 0.85% saline (0.2 ml. sera to 0.6 ml. saline). Similarly, in control sera treated with washed sheep erythrocytes (designed to control against non-specific adsorption) the anti-tissue activity was found to be reduced or removed. Subsequent studies confirmed that this phenomenon was attributable to the centrifugation procedure as little or no loss of anti-tissue activity was found in sera treated with washed sheep erythrocytes and centrifuged for 1×10^4 g minutes.

Absorption of rat serum with F_3.

Varying quantities of F_3 were mixed with constant quantities of "normal" rat serum. The mixtures were allowed to stand at room temperature for 30 minutes and overnight at 4°C. They were centrifuged for 9×10^5 g minutes and the supernates removed and tested in the complement fixation test against F_3. It was found that 80 milligrams wet weight F_3 was just sufficient to completely absorb the anti-F_3 activity from 1.0 ml. of "normal" rat serum using this modified absorption procedure.

Section (5) Heat lability of antibody in rat serum

The presence of both heat labile and heat stable complement fixing anti-tissue immunoglobulins in rat sera has been described (Sections 1 - 4). As early as 1927, Bushnell & Hudson noted that fowl serum lost some of its complement fixing activity on heating and this observation was confirmed in reports by Rice (1947) and Brumfield & Pomery (1957). These findings led to a consideration of the possibility that rat anti-sera, to a variety of antigens may contain either heat susceptible or both heat labile and heat stable immunoglobulins. The presence of such heat labile antibody would not normally be detected in standard serological tests, as sera are usually inactivated for 20 - 30 minutes at 56°C. before use. Another possibility which could account for the findings described above, was that heat induced changes in the complement fixing activity of an antibody may not necessarily be a reflection of changes in the antigen binding capacity.

An attempt was made to investigate these problems by testing rat anti-bovine serum albumin (BSA) sera, unheated and after heating at 56°C. for 30 minutes, for changes in primary binding and complement fixing activity. Rats were injected with 5 mg. alum precipitated BSA and were boosted with the same dose 12 days later. They were bled at intervals and their sera stored at -25°C. until required for testing. The antigen binding capacities of these sera were measured by the ammonium sulphate precipitation

technique of Farr (Materials & Methods p.42) and their complement fixing activity by the "micro" complement fixation test (Materials & Methods p.40). Table 11 shows the effect of heat on the antigen binding capacity and complement fixing activity of a rat anti-BSA sera. It can be seen that although heating at 56°C. does not affect the antigen binding capacity, all detectable complement fixing activity is lost. Similarly, Table 12 shows that the antigen binding capacities of sera, taken at intervals after the injection of BSA, were unaffected by heating.

Rat anti-BSA sera were fractionated by zone ultracentrifugation on sucrose density gradients and the complement fixing activity tested against BSA. There was no detectable activity in the IgG containing fractions and IgM fractions were found to be anti-complementary without the addition of BSA. However, it is now well established that IgM is more effective than IgG in fixing complement (Humphrey & Dourmashkin, 1965; Weir & Elson, 1968). Accordingly, the effect of heating on the antigen binding capacity of anti-BSA IgM fractions, prepared by sucrose density centrifugation, was studied. The results (Table 13) showed that heating had no effect on the ability of IgM fractions to bind BSA. This finding was supported by radioimmunoelectrophoretic analyses (Materials & Methods p.52) of rat anti-BSA sera. Rat anti-BSA sera were electrophoresed and precipitated by a rat anti-globulin serum selected to give a strong IgM land (see Section 6). The results (Fig. 12) showed that there was no change in the ability

Table 11

Effect on antigen binding capacity (ABC) and complement fixing activity of heating Rat anti-BSA sera

Serum	A.B.C. 33 at 0.02 g N BSA*		Reciprocal titre in C.F.T.	
	Unheated	Heated	Unheated	Heated
1	4.3	3.9	-	-
2	12	11	-	-
3	5.2	4.5	-	-
4	4.2	5.0	19	-
5	3.1	3.5	-	-
6	12.0	10.0	21	-
7	2.0	2.0	-	-
8	13	11	46	-
9	5.4	5.0	80	-

- = Negative or less than 1/5

Animals were injected with 5 ug alum precipitated BSA on day 0 and reinjected on day 12. They were bled on day 20.

Table 12

Effect of heating on antigen binding capacity of sera taken at interval after the injection of BSA

Rat		Antigen Binding Capacity at 0.02 ug N BSA*			
		Days			
		8	12	16	20
1	U	.060	.14	.19	5.4
	H	.048	.13	.20	5.0
2	U	.056	.20	.051	4.9
	H	.048	.17	.050	4.9
3	U	.040	.36	.20	3.7
	H	.036	.28	.23	4.0
4	U	.075	.260	1.4	3.8
	H	.065	.250	1.3	2.6
5	U		.080	.48	2.4
	H		.084	.46	2.4
6	U	.12	.28	1.4	
	H	.10	.25	1.1	

U=unheated, H=heated 56°C. for 30 minutes

The animals were injected with 5 mg alum precipitated BSA on day 0 and reinjected on day 12

Table 13

Effect of heating on antigen binding capacity (ABC) of whole serum and IgM fraction

	A.B.C. at 0.01 ug N BSA*		
	Whole serum	IgM U	IgM H
Rat 1	12.8	.26	.26
Rat 2	4.2	.032	.027

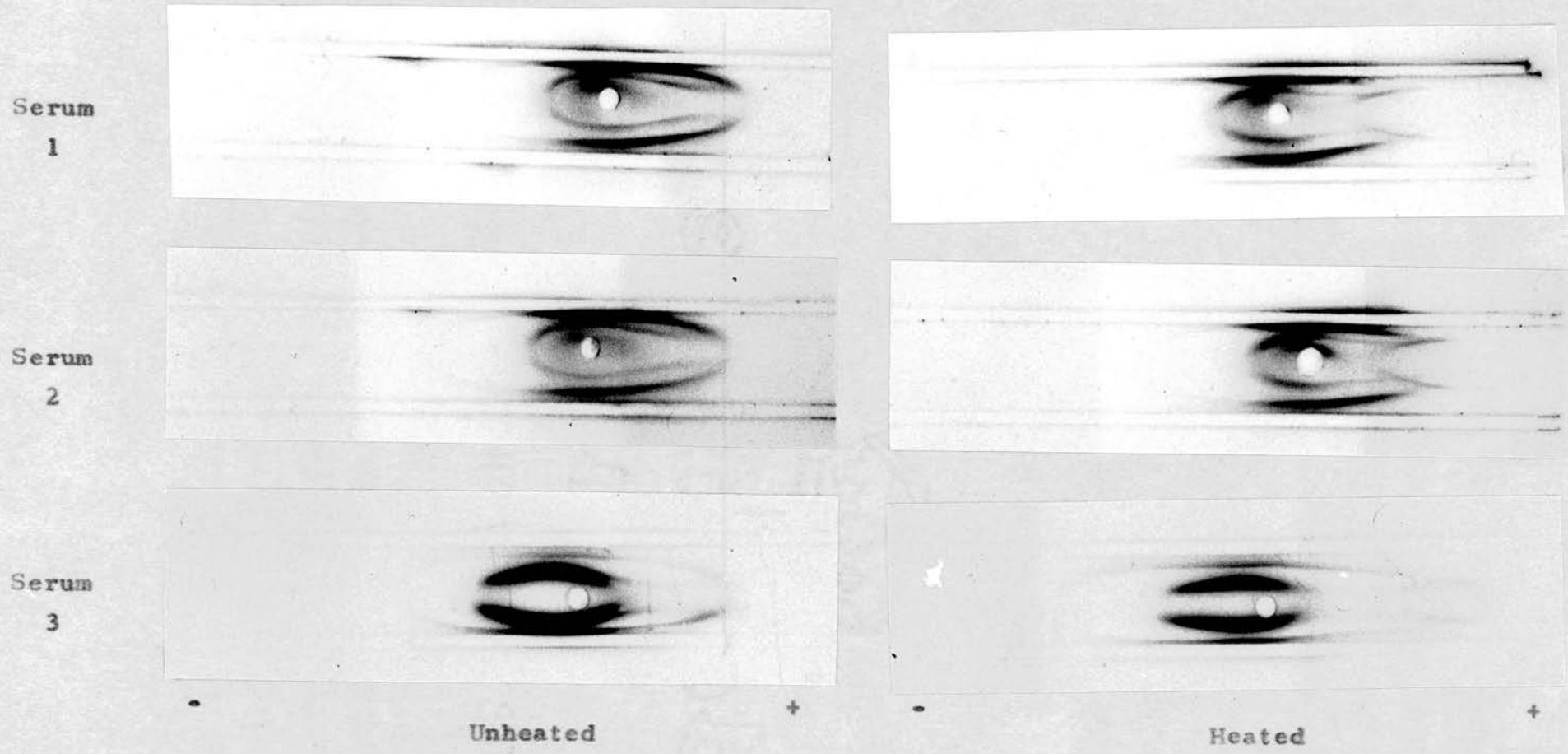


Figure 12

Radioimmunoelectrophoresis of three rat anti-BSA sera with I^{131}BSA and rabbit anti-rat globulin. Left, unheated rat sera; right, heated at 56°C. for 30 minutes. Note IgM binding of BSA after heating antiserum.

of heated anti-BSA IgM to bind BSA.

Thus it appears that the primary binding of rat anti-BSA sera (whole serum or IgM fraction) is not affected by heating the sera at 56°C. for 30 minutes, although this procedure removed the ability of the sera to fix guinea-pig complement. However, it is not clear from these results if there are two populations of antibody, one susceptible to heating and one not, or if heating has an overall effect on the antibody bringing about a reduction in its ability to activate complement.

Section (6) Uptake of serum components on sub-cellular tissue antigens

On the basis of its physico-chemical properties and its reactions in serological tests it seemed likely that the anti-tissue factor occurring in rat sera was an autoantibody. It was considered that the demonstration of a reaction between tissue antigens and the serum factor, by a technique which did not depend on a secondary manifestation of the primary interaction between antigen and antibody, would strengthen this proposition. Moreover, such a technique might show whether or not the interaction between tissue antigens and serum components was an antigen-antibody reaction or some non-specific "sponge-like" absorptive effect of the type described for leucocytes by Dausset & Colombani (1964).

Hence, the uptake of serum components on a mitochondrial preparation of rat liver (F_3) was studied by immunoelectrophoretic analysis of antisera produced by immunisation of rabbits with F_3 exposed to normal rat serum (Materials & Methods p.49). The results of these studies showed that at least 6 different serum components were taken up from the serum on the F_3 (Figs. 13 & 14). In an attempt to identify these components immuno-electrophoretic studies were carried out using the different rabbit antisera and fractions of rat sera separated on sucrose density gradients. These showed that bands 1 and 2 of Fig. 14 were the slow sedimenting globulins contained in fractions 3 and 4 and that band 3 was the

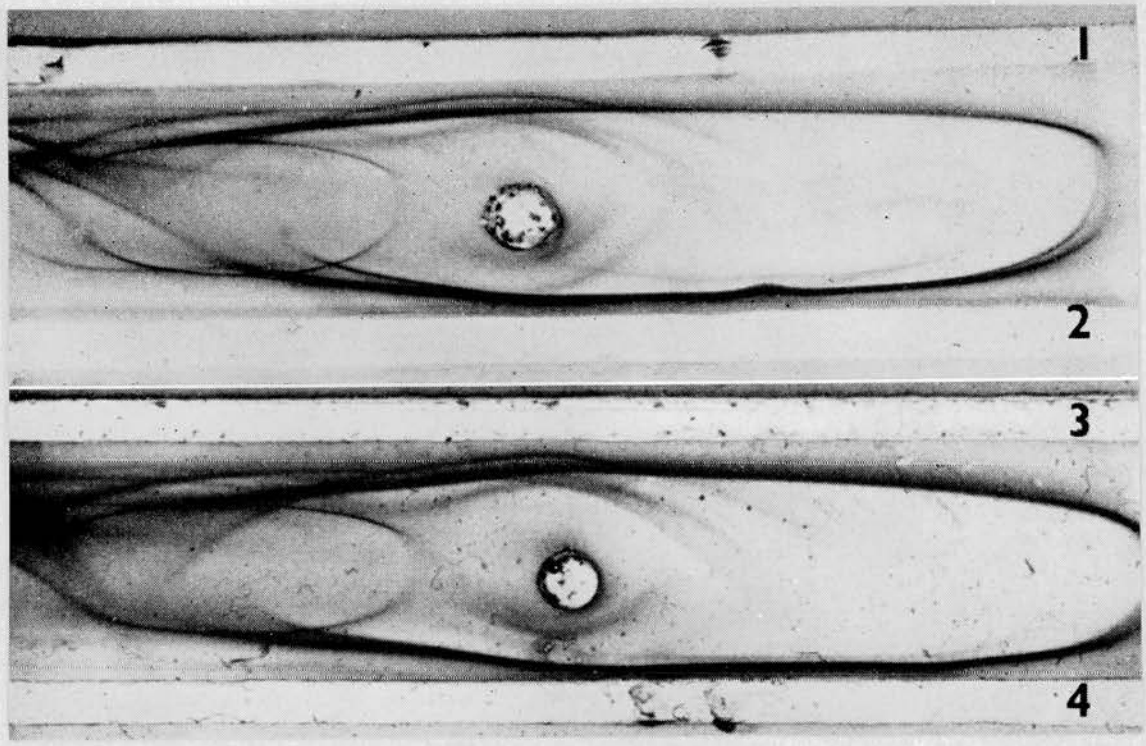


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Figure 13

Immunoelectrophoretic patterns of antisera from rabbits immunised with mitochondria (F_3) exposed to normal rat serum. Both wells contain normal rat serum. Troughs labelled 1 and 3 contain rabbit anti-whole normal rat sera. Troughs labelled 2 contain antiserum from a rabbit immunised with F_3 exposed to normal rat sera and troughs labelled 4 antiserum from a rabbit immunised with F_3 exposed to decomplemented normal rat sera.

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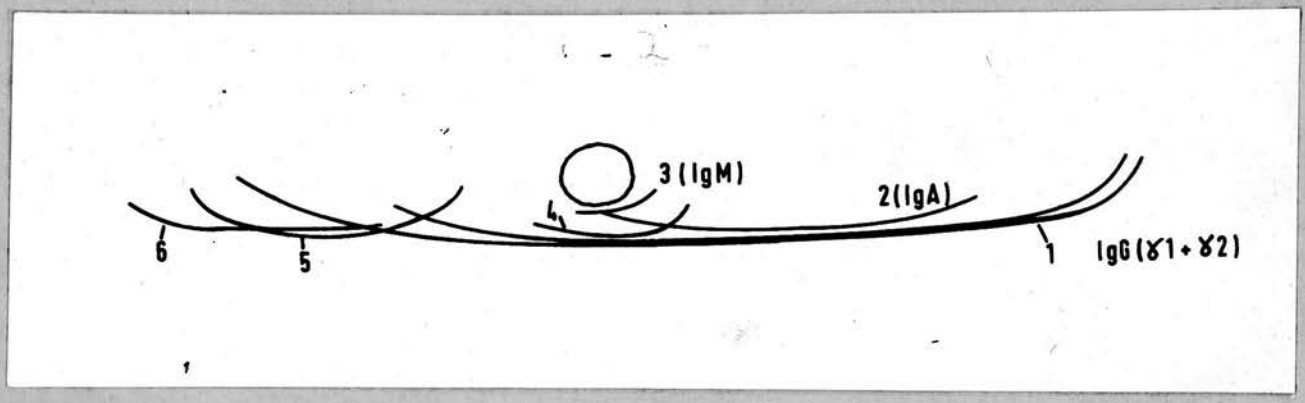

Figure 14

Schematic diagram of immunoelectrophoretic pattern of antisera from rabbits immunised with mitochondria (F_3) exposed to normal rat serum.

fast sedimenting globulin concentrated in fraction 7. Fig. 15 shows the pattern obtained with the sucrose density gradient fractions 4 - 8 of normal rat serum. On the basis of tests carried out in the standardisation of the procedure (Weir et al, 1966; Coghlan & Weir, 1967), which showed the concentration of the IgG and IgA globulin in fractions 3 and 4 and the IgM globulin in fractions 6 and 7, these results would be consistent with the labelling of the precipitin bands shown in Fig. 13. Moreover, band 1 appears to be identical to that designated IgG (γ1 and γ2) by Nussenweig & Binaghi (1965) and bands 2 and 3 to those designated IgA and IgM by Arnason, De Vaux St. Cyr & Relyveld (1964) and Binaghi & de Merlo (1966). The coating of F_3 with decomplemented sera (Fig. 13) showed that components 4 and 6 were removed indicating that these were complement components. Decomplemented rat sera were prepared by treating normal rat sera with excess BSA-rabbit anti-BSA immune precipitate prepared at equivalence as described in the Materials and Methods (p.69). This procedure was found to remove all the complement activity as judged by the effect of the absorbed sera on sensitised sheep erythrocytes. It was found to have no effect on the anti-tissue activity.

The possibility arose that this phenomenon was merely due to histocompatibility isoagglutinins. This was rendered unlikely by the finding of antibodies against the same rat immunoglobulins and complement components in anti-sera of rabbits injected with

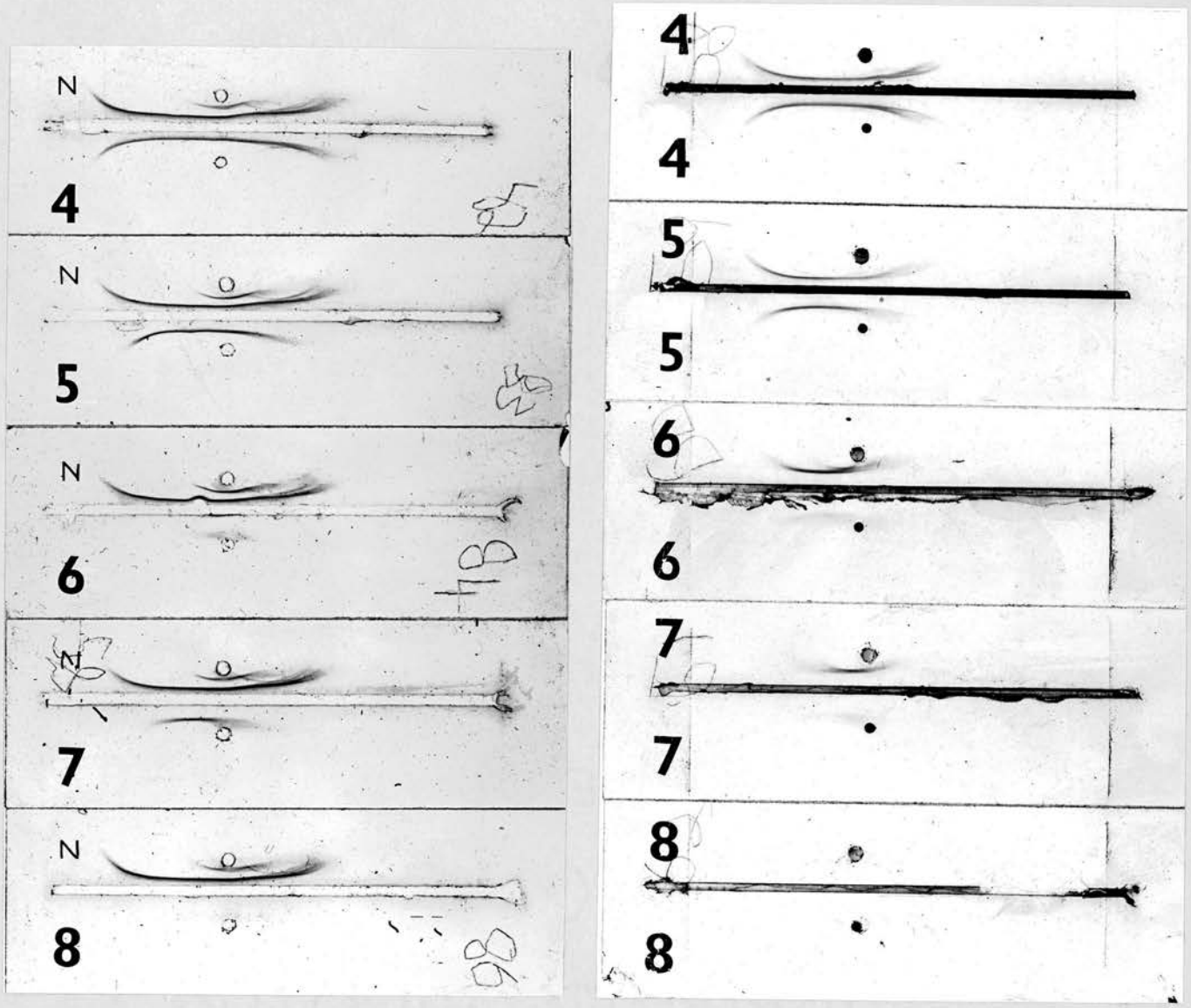

Figure 15

Immunoelectrophoretic pattern obtained with the rabbit antisera shown in Fig. 13 set up as indicated.

The wells labelled N contained normal rat serum and the other wells are labelled according to the sucrose density gradient fractions which they contain.

F_3 coated with autologous serum (Fig. 15a).

No antibodies against rat serum components were detected in antisera of four rabbits injected with F_3 alone.

An experiment was set up to determine whether there was any difference in the uptake of serum components by F_3 from "normal" rat sera and sera taken four days after CCl_4. A rat was bled and injected with CCl_4. Four days later it was bled again. These sera were taken and tested in the complement fixation test against F_3 and the results are shown in Table 14.

Table 14

	Titres in Complement Fixation Test	
	Unheated	After heating at 56°C. for 30 mins.
"Normal" Serum	32	< 2
Sera taken 4 days after CCl_4	512	256

F_3 was exposed to the normal serum and to the serum taken four days after CCl_4. The preparations of coated F_3 were injected into separate rabbits and the anti-sera tested for the presence of antibodies against rat serum components by immunoelectrophoretic analysis. The results can be seen in Fig. 16.

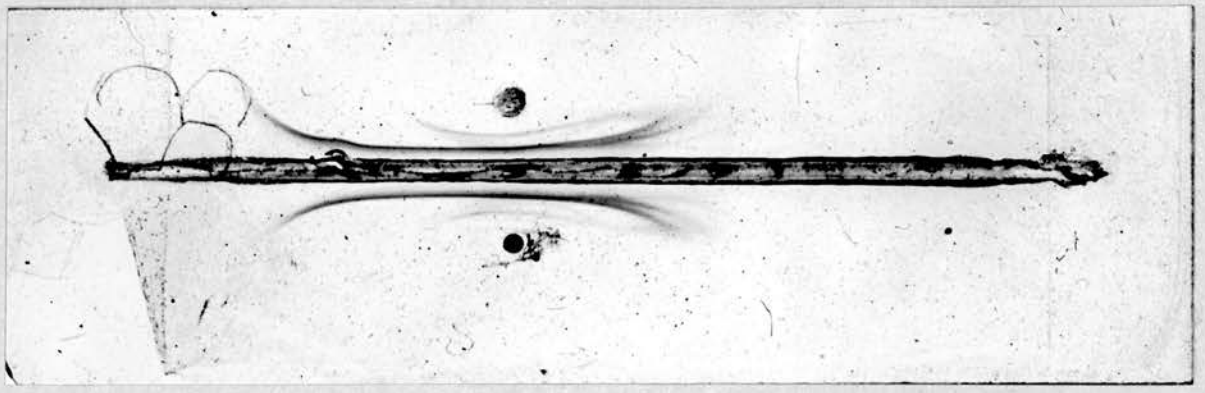

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Figure 15 a

Immunoelectrophoretic pattern of antiserum from a rabbit immunized with rat mitochondria (F_3) exposed to autologous normal rat serum. The well contained normal rat serum.

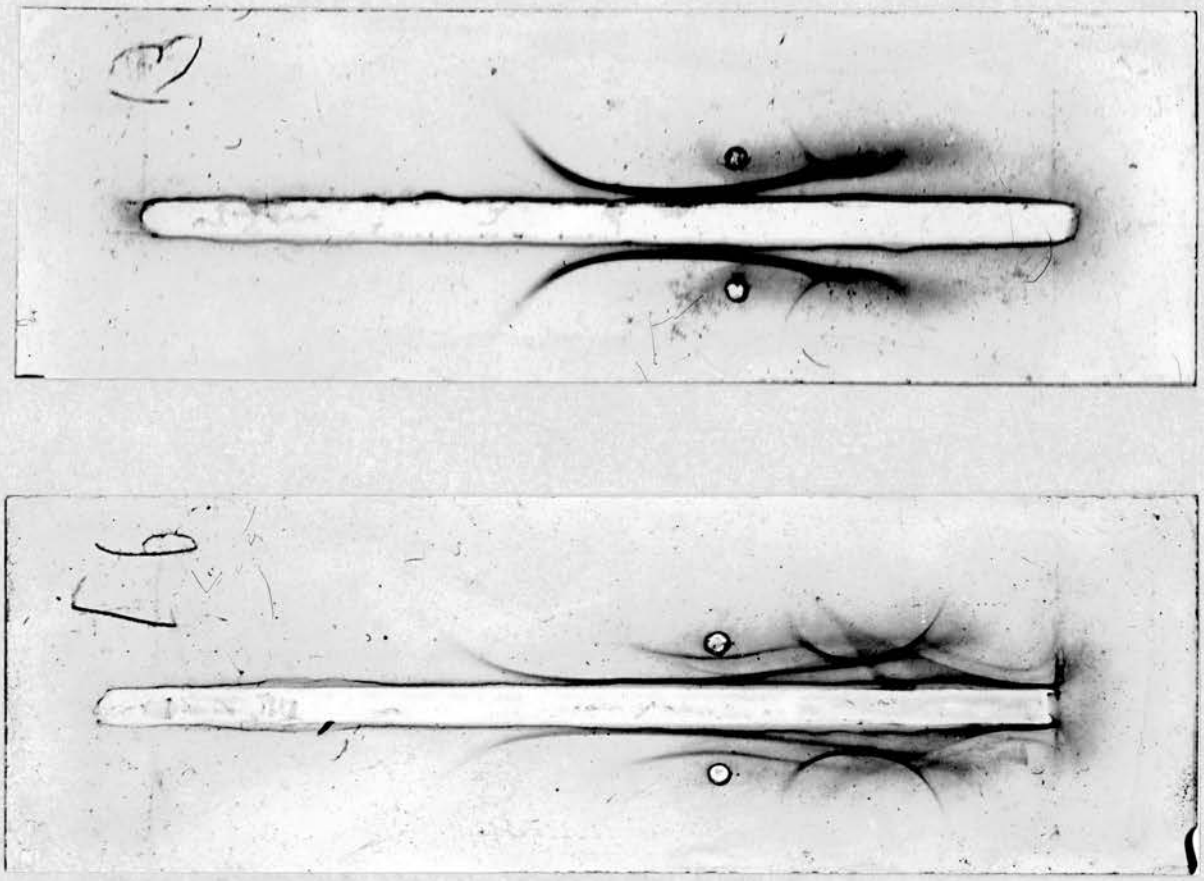

Figure 16

Immunoelectrophoretic pattern obtained with the serum of a rabbit immunised with rat mitochondria (F_3) exposed to the serum of a normal rat (upper plate) and with the serum of a rabbit immunised with the same F_3 exposed to serum from the rat taken 4 days after CCl_4 (lower plate). The wells contained normal rat serum.

Uptake of Individual Immunoglobulin classes

The results described above indicated that there is selective uptake of immunoglobulins and complement components from normal rat serum by F_3. However, the possibility could not be excluded that the uptake of IgM, IgA and IgG was an apparent effect due to the uptake of one particular class of immunoglobulin. If this were the case, then the rabbit anti-sera would contain antibodies reactive with light chain determinants of all classes of immunoglobulins. The purpose of the following studies was to determine whether each individual class of immunoglobulin was in fact taken up by F_3.

Preliminary investigations, in which the sera of a rabbit injected with F_3 exposed to a highly purified IgG preparation of "normal" rat sera (Materials & Methods p.59) was analysed by immunoelectrophoresis, suggested IgG was not taken up by F_3. A similar experiment was designed, in which various preparations of F_3 were made up as shown below and injected into guinea pigs and rabbits. The work of Binaghi, Oriol & Boussac-Aron (1967) suggested that guinea pigs responded only to the Fc fragments of heterologous immunoglobulins. If this were the case, then guinea-pigs should produce class specific anti-sera against rat immunoglobulins.

The highly purified IgG was prepared by salt fractionation of normal rat sera followed by batch processing with DEAE and DEAE chromatography. The IgG preparations were found to be free from

other contaminating serum proteins as judged by immunoelectrophoretic analysis with rabbit anti-rat serum. The IgM was prepared by Sephadex chromatography (Materials & Methods p.53) and was contaminated with α2 macroglobulins.

Preparation	F_3 coated with	Preparation injected into	
		Guinea pigs	Rabbits
1	Normal rat sera	2	1
2	Normal rat sera	4(1 died)	1
3	Highly purified IgG	2(1 died)	1
4	Highly purified IgG	2(1 died)	1
5	Highly purified IgG		2
6	Normal rat IgM	2(1 died)	2
7	F_3 alone	2	

The results of immunoelectrophoretic analysis of the resultant anti-sera against normal rat sera are summarised below.

Preparation	1	2	3	4	5	6	7
	F_3+NRS	F_3+NRS	F_3+IgG	F_3+IgG	F_3+IgG	F_3+IgM	F_3
Guinea pigs	-	-	+	-		+	-
	-	-					-
		+					
Rabbits	+	+	+	$\pm$	-	+	
					-	+	

+ = distinct precipitin bands $\pm$= faint precipitin bands

- = no precipitin bands

Of the surviving guinea pigs injected with F_3 coated with NRS, only one was found to produce antibodies against rat serum components. The immunoelectrophoretic pattern obtained with this sera, is shown in Fig. 17. An IgM band can be clearly seen although there is no IgG band. The finding that the sera of rabbits injected with the same preparations contained antibodies against rat serum components showed that rat serum components had been taken up on F_3.

Fig. 18 shows the immunoelectrophoretic pattern of the rabbit injected with preparation 3 (F_3+IgG). Only an IgG band can be seen. The sera of a guinea pig injected with preparation 3 showed a similar pattern on immunoelectrophoretic analysis. The sera of the rabbit injected with preparation 4 (F_3+IgG) showed a barely detectable IgG band. No precipitin bands were seen on immunoelectrophoretic analysis of serum from the guinea pig injected with preparation 4 (F_3+IgG) or sera from the rabbits injected with preparation 5 (F_3+IgG).

Fig. 19 shows the immunoelectrophoretic patterns obtained with the sera of animals injected with preparation 6 (F_3+IgM). The rabbit antisera can be seen to give both IgM and IgG bands. In contrast the guinea pig antisera gave only an IgM immunoglobulin band.

Effect of heating on uptake of serum components by F_3

It has been shown that the complement fixing activity of rat

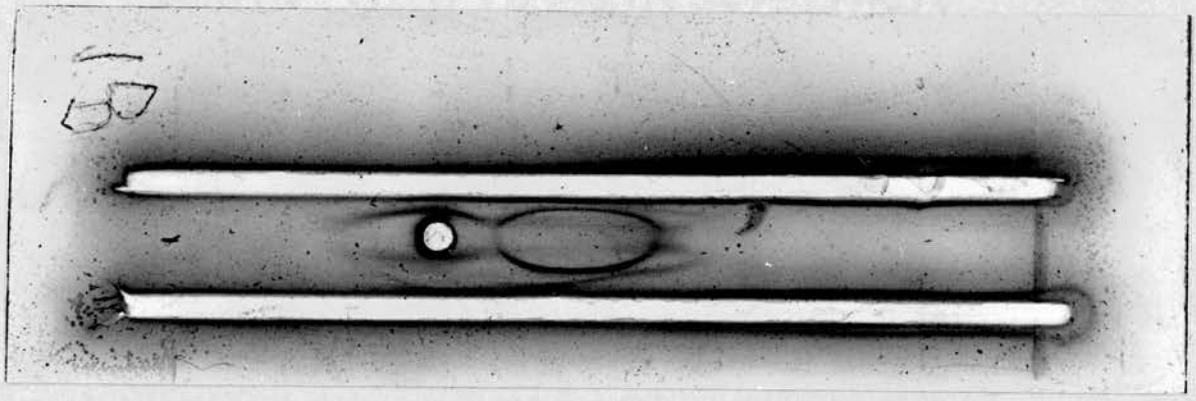

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Figure 17

Immunoelectrophoretic pattern obtained with the sera of a guinea pig immunised with rat mitochondria (F_3) exposed to normai rat serum.

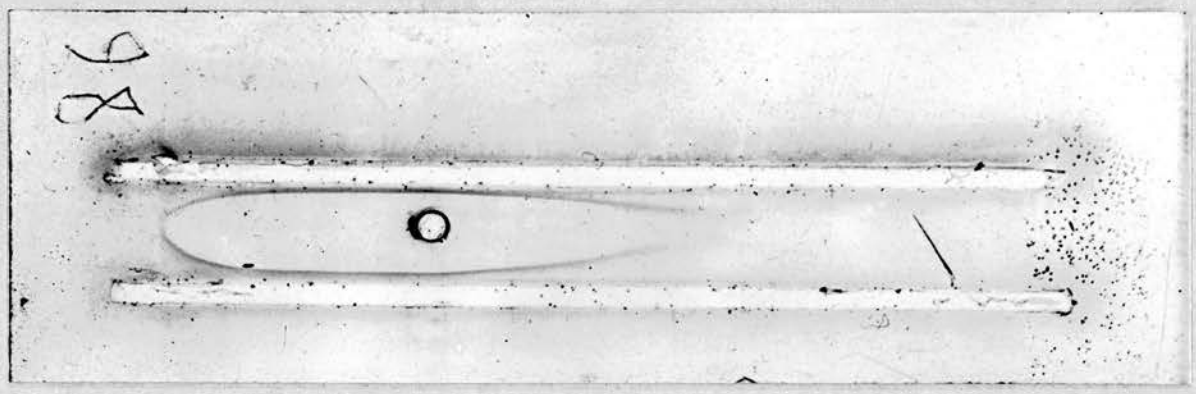

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Figure 18

Immunoelectrophoretic pattern obtained with antisera from rabbit immunised with rat mitochondria (F_3) exposed to a purified preparation of normal rat IgG. The well contained normal rat serum. Note prominent IgG bands.

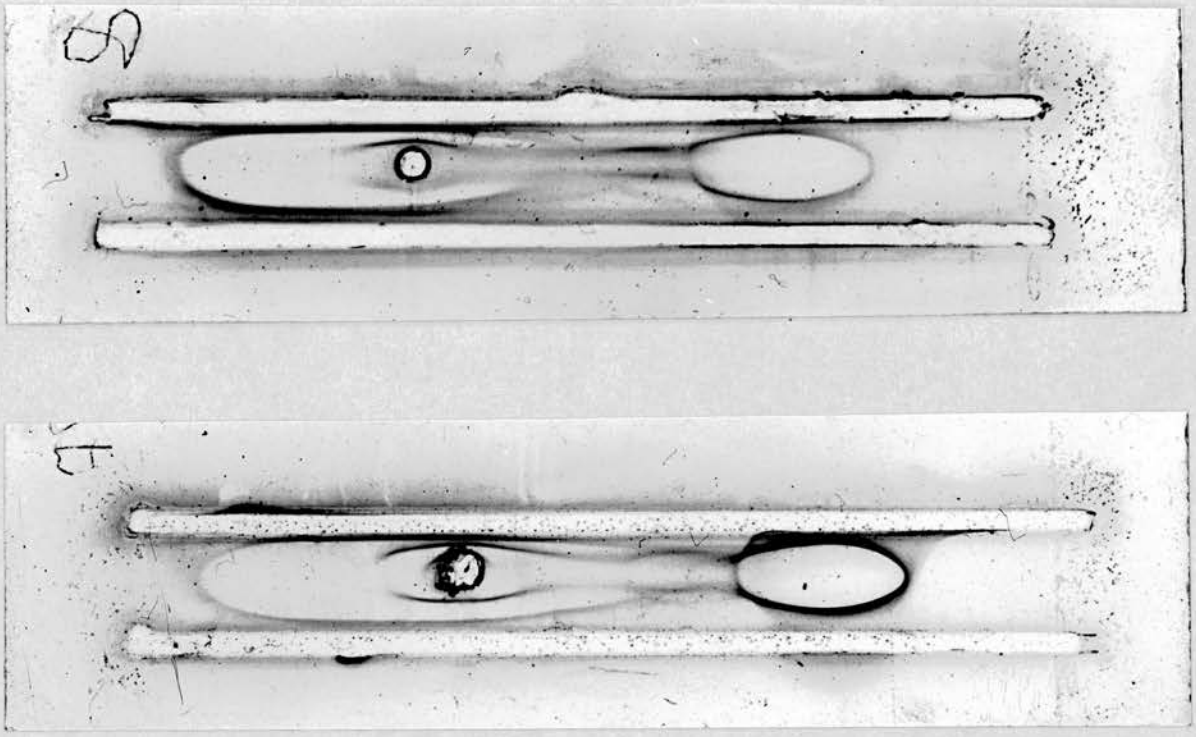

Figure 19

Immunoelectrophoretic patterns obtained with antisera from rabbits (above) and a guinea pig (below) immunised with rat mitochondria exposed to an IgM preparation of normal rat serum. The wells contained normal rat sera. Note the absence of an IgG band in the pattern obtained with the guinea pig antisera.

anti-BSA sera is diminished by heating the antisera at 56°C. for 30 minutes although this procedure does not affect its antigen binding capacity. These results suggested that the primary binding of the anti-tissue serum factor may not be affected by heating "normal" rat sera at 56°C. for 30 minutes.

A normal rat serum pool was obtained and divided into aliquots. One aliquot was heated at 56°C. for 30 minutes. F_3 was treated with the unheated aliquot and similarly with the heated aliquot. The preparations of coated F_3 were injected into separate rabbits and the resultant antisera tested by immunoelectrophoretic analysis against normal rat serum. The results (Fig.20) showed that the binding of the naturally occurring anti-mitochondrial immunoglobulins was not destroyed by heating "normal" rat serum at 56°C. This contrasted with the complement fixing anti-mitochondrial activity which was destroyed by heating the sera at 56°C.

Attempt to detect in vivo uptake of serum components

An attempt was made to detect in vivo binding of serum components by damaged tissue. Three rats were injected with CCl_4 and killed at 4, 24 and 96 hours respectively. Their livers were removed and a mitochondrial preparation (F_3) made from each. The preparations were injected into separate rabbits and the resultant antisera tested for antibodies against rat serum components by immunoelectrophoretic analysis. No antibodies against rat serum components were detected.

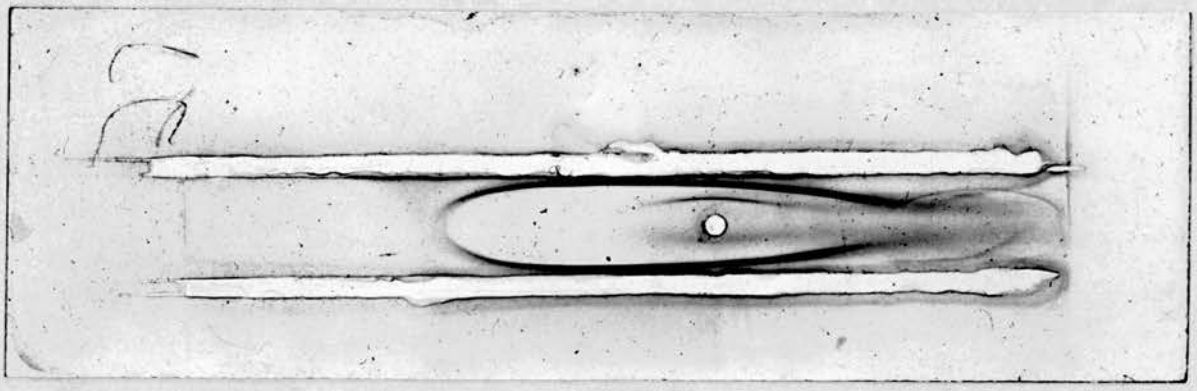

Figure 20

Immunoelectrophoretic pattern of antisera from rabbits immunised with rat mitochondria (F_3) exposed to unheated or heated (56°C for 30 mins) normal rat sera. The well contained normal rat serum. The upper trough contained antisera from rabbit immunised with F_3 exposed to unheated rat sera and the lower trough antisera from rabbit immunised with F_3 exposed to heated rat sera.

Section (7) Role of Anti-tissue Immunoglobulins

The work described in this thesis suggests that complement fixing autoantibodies against sub-cellular tissue components are present in most normal rat sera. This work also indicates that the antibodies induced by toxic liver damage (Weir, 1963, 1966) are an enhanced level of these naturally occurring antibodies since both appear to be IgM in type and to be directed against the same antigenic determinants. No evidence is available which suggests that these antibodies play any part in causing cell damage (Weir, 1963). Moreover a number of workers (see Introduction p. 29) have postulated that such immune reactions against "self material" may occur and may have some physiological activity. Thus efforts have been directed towards defining a physiological role for immunoglobulins of this type, perhaps in initiating and facilitating clearance of tissue breakdown products.

Chemotaxis of Polymorphs induced by Tissue antigens and rat serum

Antigen-antibody complexes have been shown to activate a factor in "normal" sera which has a direct chemotactic influence on polymorphonuclear leucocytes (PMN)(Boyden, 1962). This factor has been identified as the C'_5, C'_6 and C'_7 components of complement (Ward, Cochrane & Muller-Eberhard, 1965, 1966). These observations together with those described above, suggested that the complement fixing anti-tissue autoantibody may be implicated in the removal of tissue breakdown products by rendering them

attractive to PMN. This hypothesis was tested by measuring the effect of the interaction of anti-tissue autoantibody and subcellular components on polymorphonuclear leucocyte migration by Boyden's two compartment chamber technique (Materials & Methods p. 64). This method utilizes chambers which are separated into two compartments by a "millipore" membrane through which PMN can pass only by active migration. The PMN were placed in the upper compartment and the test substances in the lower. The number of PMN migrating through the membrane was determined by counting the number of PMN attached to the lower surface of the membrane.

The effect of mixing normal rat serum (NRS) with a mitochondrial preparation of rat liver (F_3) is shown in Table 15. It can be seen that normal rat serum mixed with F_3 induced PMN migration whereas the reagents alone have only a slight effect.

Preliminary investigations revealed a number of factors which appeared to affect this phenomenon. It was found that the polymorph suspension must not be contaminated with blood. Storing serum at -25°C. increased the chemotactic effect of serum alone. The proportion of normal serum to F_3 appeared to be important. The phenomenon was most marked when the serum and F_3 were mixed at "optimal proportions" (see Results p.84). Finally, the phenomenon appeared to be most consistent if PMN from a single rat were used in an experiment.

Heating the serum at 56°C. for $\frac{1}{2}$ hour inhibited the migration of PMN (Table 16). However, if the reagents were incubated prior

Table 15

Effect of mixing normal rat serum (N.R.S.) and F_3 on PMN migration

Expt.	No. of cells/field (Mean of 3 tests) N.R.S. + F3	N.R.S.	F3
1	43	8	15
2	410	19	3
3	53	6	8
4	346	24	24

In each experiment 0.2 ml. N.R.S. and/or 0.2 ml. F_3 was made up to 1 ml. with the medium

Table 16

Effect on PMN migration of heating "normal" rat serum (NRS) at 56°C. for 30 mins. prior to and after mixing with F_3.

The preparations were treated as shown and were tested as before

Number of Cells/field (Mean of 3 tests)

Expt.	NRS+F_3	NRS	NRS+F_3 incubated for 3 hours and then heated at 56°C. for 30 mins.	NRS incubated for 3 hours and then heated at 56°C. for 30 mins.	NRS (heated at 56°C. for 30 mins.) + F_3
1	204	53	197	60	
2	110	20	110	13	
3	110	9	123		
4	269	3			2

to heating the migration was unaffected.

An attempt was made to compare the PMN response to F_3 and normal serum with a known antigen antibody reaction. The results are shown in Table 17.

The effect on PMN migration of mixing NRS with other subcellular fractions is shown in Table 18.

An attempt was made to compare polymorph migration to a mixture of F_3 and normal rat serum with that to a mixture of F_3 and serum from the same animal taken 4 days after the injection of CCl_4. The results are shown in Table 19.

Response of Macrophages to tissue breakdown products

It has been proposed that the cellular response to inflammation occurs in two stages; the first being emigration of PMN and the second emigration of mononuclear cells (Page, 1964). Paz and Spector (1962) have challenged this concept and have contended that both cell types begin emigration at the same time in response to the same stimuli. It seemed necessary, therefore, to ascertain whether serum-tissue antigen mixtures were chemotactic for mononuclear cells.

Preliminary investigations were carried out using Boyden's chamber technique with millipore membrane's of 3μ pore size and suspension consisting mainly of macrophages. The suspension was obtained by washing the intraperitoneal cavity (see Materials & Methods p.66) of either a "normal" rat or a rat injected with a

Table 17

Number of cells/field (Mean of 3 tests)					
Expt.	Serum + F_3	F_3	Serum	Serum + BSA	BSA
1	154	10	10	92	6
2	78	3	5	17	1
3	272	4	20	97	10

The serum was a pool of rat anti-BSA

Table 18

Subcellular Fraction		No. of cells/field (Mean of 3 tests) Fraction + N.R.S.	Fraction Alone
F_1	nuclei + cell debris	96	33
F_3	mitochondrial	52	12
F_4 & F_5	mitochondrial	49	7
F_6	microsomal	20	1
F_7	cell sap	29	5
N.R.S. alone		19	

Table 19

Number of cells/field			(Mean of three tests)	
NRS + F_3	NRS	F_3	CCl_4D_4 Serum + F_3	CCl_4D_4 serum
18	5	7	26	18

glycogen suspension 4 days previously. The results of these investigations (Tables 20 and 21) showed that macrophages appeared to be attracted by particulate tissue material rather than by a serum-tissue antigen interaction.

Table 20

Effect of mixing normal rat serum (NRS) and rat liver homogenate (WLH) on macrophage migration.

	Number of cells / field		(Mean of 3 tests)
Expt.	WLH + NRS	WLH	NRS
1	6	18	2
2	13	14	1
3	16	20	1
4	4	6	3

Table 21

Ability of various rat liver fractions to induce macrophage migration

	Number of cells/field (Mean of 3 tests)					
F_1	F_2	F_3	F_4	F_5	F_6	F_7
8	8	1	1	1	1	1

This conclusion was supported by the results shown in Table 22.

A PMN suspension was obtained and divided into 2 aliquots. One aliquot was ultrasonically disintegrated and fractionated into particulate and supernate fractions by centrifugation for 5×10^3 g minutes. The effect of the whole PMN suspension and the particulate and supernate fractions of the disintegrated cells on macrophage migration was tested. It can be seen that the particulate material from disintegrated polymorphs has the greatest influence on macrophage migration.

Table 22

	Number of cells/field (Mean of 3 tests)		
Medium alone	Whole PMN	Particulate Fraction of PMN	Supernate Fraction of PMN
1	5	15	1

Attempts to demonstrate opsonising effect of anti-tissue immunoglobulins

Antibodies are known to opsonise the phagocytosis of a variety of materials by the cells of the reticulo-endothelial system (RES) (Stiffel et al, 1964; Vaughan, 1965). It has been shown that a mitochondrial fraction of rat liver treated with normal rat serum selectively takes up immunoglobulins, including the IgM induced by tissue breakdown products together with complement components it has activated. These findings suggested that the anti-tissue immunoglobulins may facilitate phagocytosis

of tissue breakdown products by the cells of the RES. A number of experiments were set up to test this hypothesis and although none yielded conclusive results, they suggested an approach to further work.

Preliminary investigations on the effect of "normal" rat serum on the uptake of rat liver mitochondria by peritoneal macrophages suggested that "normal" serum may have an oponizing effect*. Macrophages were incubated with a fresh mitochondrial preparation of rat liver (F_3) alone and in the presence of normal rat serum. The uptake of mitochondria was estimated by microscopic examination of the macrophages. The mitochondria were stained by histochemical techniques which depended on the localisation of succinic dehydrogenase and alkaline phosphatase in mitochondria. However this technique was difficult to quantitate and no clear cut difference could be said, with certainty, to exist between controls and tests.

Similar studies were performed with I^{131} labelled F_3. These failed due to the difficulty found in separating macrophages and unphagocytosed $I^{131}F_3$ for counting purposes.

It is well established that macrophages isolated from different parts of the body utilize different metabolic pathways and differ in their phagocytic discrimination (Gesner & Howard, 1967). An attempt was made, therefore, to examine the localisation of subcellular components after their injection into the blood stream. Rats were injected intravenously with $I^{131}F_3$ and killed 5 minutes

* These experiments were performed in conjunction with Mr.J.Boulton and Dr. A.E. Stuart, to whom I am indebted for carrying out the staining procedures.

and 1 hour after injection. Portions of various organs were removed, weighed and the number of radioactive disintegrations/ unit time emitted by each portion was counted in a scintillation spectrometer. The results (Table 28) showed that the liver rapidly took up a considerable proportion of the injected $I^{131}F_3$. This suggested that the isolated perfused liver may be a suitable organ to study the effects of serum on the phagocytosis of sub-cellular components.

Table 23

Localisation of I^{131} F_3 at intervals after intravenous injection

Rat	Organ	% of total radio-activity injected	counts/gm tissue per unit time
1 Killed 5 mins. after injection.	Liver	45.3	2924
	Spleen	1.5	929
	Lung	3.2	1211
	Heart	3.6	1907
	Thyroid	$\leq .1$	70
2 Killed 1 hour after injection.	Liver	11.3	604
	Spleen	1.5	377
	Lung	1.6	615
	Heart	.7	362
	Thyroid	.7	405

Section (8) Evasion of Tolerance by Tissue antigens

The finding that rats produced anti-liver antibody early in life (Results Section (1)) suggested that the apparent failure of rats to develop a normal tolerance to the corresponding tissue antigen was not due to these antigens being "hidden" from the antibody forming cells during early life as has been suggested by Burnet (1963). Further evidence contradictory to Burnet's hypothesis was provided by the work of Weir & Pinckard (1967) who showed that rats subjected to an intensive injection schedule of rat liver antigens from birth, did not develop immunological tolerance to these antigens as judged by their ability to produce anti-liver antibodies at 5-6 weeks after birth. The question remains as to how and why these antigens evade the normal tolerance inducing mechanism.

There is evidence that a number of factors may hinder the induction of immunological tolerance (see Introduction p.6). On the basis of the work described in this thesis, some of these factors were thought likely to affect the induction of tolerance to tissue antigens.

(a) The finding (Section 4) that the anti-tissue antibody was directed against sedimentable components of tissue homogenates suggested that the failure to become tolerant to these antigens was in some way associated with the particulate nature of the antigen. Conversely it would be expected that rats would be unresponsive or hyporesponsive to soluble tissue antigens.

(b) The finding that rats produced anti-tissue antibody early in life (Section 1) suggested that the immunological system may mature earlier in ontogenesis to these antigens than to others. Another possibility raised by this finding was that (c) passage of maternal antibody via the yolk sac and colostrum may occur and influence the induction of tolerance with respect to these antigens. The following experiments were designed to test these hypotheses:-

Effect of alum on Induction of Tolerance to bovine serum albumin (BSA)

Pinckard (1967) found that less than 1% of I^{131} BSA was removed by washing alum precipitated BSA with saline. This suggested that predominantly particulate alum precipitated BSA and "soluble" BSA may be suitable antigens to use in assessing the effect of the physical state of an antigen on the induction of neonatal tolerance. Rats were injected intraperitoneally within 24 hours of birth with freshly prepared thrice washed (in 0.85% saline) alum precipitated BSA. Littermate controls were injected with a solution of BSA (NBA) and aggregate free BSA (CBA). The aggregate free BSA was prepared by centrifuging 4 ml. of a BSA solution in a polypropylene tube (5 x 1.1 cm) for 6×10^6 g minutes. The top 2 ml. was removed and used immediately. Other littermate controls were injected with alum alone or saline. They were challenged with alum precipitated BSA at 5 weeks and their serum taken and tested for its ability to bind BSA using

the ammonium sulphate precipitation technique of Farr (see Materials & Methods p.42). The results (Table 24) showed that alum precipitated BSA was more effective in inducing tolerance than either NBA or CBA.

Immunogenicity of Soluble Tissue antigens

The possibility that rats are unable to react immunologically to their own soluble tissue antigens was tested by measuring the ability of adult rats to respond to the injection of a soluble preparation of tissue antigens presented in various forms. A soluble extract (F_7) of liver homogenate was prepared by differential centrifugation (see Materials and Methods p. 62). This preparation was treated in various ways with a view to increasing its "immunogenicity" and injected into groups of animals as shown below:-

Group	Number of rats:group	Each rat injected intraperitoneally at 14 day intervals with
A	6	0.5 ml. untreated F_7.
B	5	0.5 ml. F_7 emulsified with 0.5 ml. Freund's complete adjuvant (Difco).
C	5	0.5 ml. F_7 mixed with 0.5 ml. alum.
D	5	0.5 ml. F_7 dialysed overnight at 4°C. against phosphate buffered saline.
E	5	0.5 ml. dialysed F_7 emulsified with 0.5 ml. Freunds complete adjuvant.

The rats were bled at intervals and their sera taken and tested in the complement fixation (Materials and Methods p.38)

Table 24

Effect of Alum on Induction of Tolerance to B.S.A. in neonatal rats

Antigen Binding Capacity at 0.02 ug N B.S.A.*							
5 mg. alum precipitated B.S.A.		5 mg. normal B.S.A. (NBA)		5 mg. aggregate free B.S.A. (CBA)		Control (alum alone or saline)	
Day 12	Day 20	Day 12	Day 20	Day 12	Day 20	Day 12	Day 20
<.01	<.01	.14		.07	1.8	.18	
<.01	<.01	.15	>25	.12	.56	.14	6.3
<.01	<.01	.13		<.01	.09	<.01	
<.01	<.01	.18		.11		.20	11.5
<.01	<.01			.03		<.01	.20
.17	10.3					.14	9.3
<.01				.10	.44	.26	
.01						.23	1.7
<.01						.32	5.0
.25	20.0					.18	
<.01	.08					.18	5.4
<.01						.19	10.6
<.01						.12	>25
<.01	<.01					.12	9.5
<.01						.41	
						.25	7.0

Rats were injected within 24 hours of birth as shown above. They were challenged with 5 mg. alum precipitated B.S.A. 5 weeks after birth and boosted, after bleeding, 12 days later. They were bled finally after a further 8 days.

against the original F_7 and dialysed F_7. The results (Tables 25 and 26) showed that rats were able to respond to the injection of F_7. Moreover the sera of some "normal" rats fixed guinea pig complement in the presence of F_7.

Some of the sera were tested, before and after heating at 56°C. for 30 minutes in the complement fixation test against F_7. The results (Table 27) showed that the complement fixing activity was reduced or removed by heating.

Four of the rats were killed and their liver removed, homogenised, centrifuged and the supernates taken as F_7. Their sera were tested in the complement fixation test against these autologous preparations of F_7 and against homologous F_7. The results (Table 28) showed that the sera reacted with autologous material.

Three positive sera were fractionated by some centrifugation in sucrose density gradients (Materials & Methods p. 56). Nine fractions were taken and tested in the complement fixation test against F_7. The results showed that the complement fixing activity was localised in each case, in fractions 6 and 7 (the IgM containing fractions). This result, together with those described above, suggests that this activity, like that described previously, is due to an IgM autoantibody.

It appeared necessary to confirm that the antigen (in vitro) was soluble. Preparations of F_7 and dialysed F_7 were centrifuged for a total of 1.8×10^7 g minutes (3 times 6×10^6 g mins).

Table 25

Titres in complement fixation test of rat sera against the soluble fraction of rat liver (F_7)

Rats were injected with various forms of F7 on days 0, 14 and 28

Day	0	4	7	10	14	18	22	28	32	38	Rat	Group
	-	64	-	16	32	32	32	-	-	AC	a	A
	-	64	64	32	64	128	-	-	-	32	b	(F_7
	32	64	256	128	128	256	64	64	64	≠	c	alone)
	-	64	-	-	-	-	8	-	32	32	d	
	-	32	-	-	16	-	-	-	-	-	e	
	16	AC 64	32	64	32	32	32	128	32	≠	f	
	16	512	256	?	32	16	64	32	16	≠	a	B
	16	-	-	32	32	64	32	32	32	32	b	(F_7 in
	-	-	16	16	-	AC 64	128	256	16	32	c	Freunds Complete Adjuvant)
	-	-	-	-	-	-	-	-	-	-	d	
	-	512	64	64	128	AC 64	AC 32	-	-	32	f	
	-	32	-	8	-	16	8	-	16	8	a	C
	-	32	-	-	-	-	16	-	32	≠	b	(Alum pre-
	-	128	16	32	-	32	≠				c	cipitated F_7)
	AC 16	AC 64	AC 64	AC 128	AC 64	AC 64	AC 64	AC 64	AC 64	AC 128	d	
	AC 16	-	-	-	-	128	256	32	64	32	f	

- = negative or <4 ≠ = animal died AC = Anti-complimentary

Table 25

Day 0	4	7	10	14	18	22	28	32	38	Rat	Group
-	128	32	-	-	16	8	-	-	-	a	D
-	-	-	-	-	-	-	-	-	-	b	(Dialysed
-	-	-	-	-	-	16	-	-	-	c	F_7)
-	32	8	-	-	-	-	-	16	8	e	
-	-	64	32	-	16	16	-	32	-	f	
-	-	?	-	-	-	-	-	-	32	a	E
-	AC 128	64	32	32	32	16	16	-	16	c	(Dialysed F_7 in
-	AC 64	-	-	-	-	-	↯			d	Freunds Complete Adjuvant)
-	-	-	-	-	-	-	-	-	-	e	
8	64	64	64	64	16	32	16	-	8	f	

- = negative or <4 ↯ = animal died AC = Anti-complementary

Table 26

Titres in complement fixation test of rat sera against dialysed F_7.
Rats were injected with various forms of F_7 on days 0, 14 and 28.

Day 0	4	7	10	14	18	22	28	32	38	Rat	Group
8	32	8	8	16	16	16	16	16	AC 16	a	A
16	32	32	32	64	16	32	32	32	32	b	(F_7
32	128	128	64	128	64	32	32	↯		c	alone)
16	32	-	16	16	16	16	16	16	16	d	
8	32	-	-	32	-	16	8	32	16	e	
32	AC 64	64	32	32	32	32	128	32	↯	f	
32	256	128		32	16	32	32	↯		a	B
16	-	-	16	32	32	32	32	32	16	b	(F_7 in
16	8	32	16	8	AC 64	64	128	16	16	c	Freunds Complete Adjuvant)
-	-	-	4	-	-	8	-	-	↯	d	
16	512	64	64	64	AC 32	AC 32	8	-	32	f	
-	32	16	8	16	32	16	8	32	16	a	C
-	-	-	-	-	-	16	-	16	↯	b	
-	64	16	32	-	16	↯				c	(Alum precip-
AC 16	AC 32	AC 32	AC 64	AC 32	AC 32	AC 32	AC 32	AC 64	AC 32	d	itated F_7)
AC 16	?	16	8	8	64	32	16	32	32	f	

- = negative or <4 ↯ = animal died AC = Anti-complementary

Table 26

Day 0	4	7	10	14	18	22	28	32	38	Rat	Group
8	128	64	16	8	32	8	8	16	16	a	D
8	-	-	16	8	16	16	-	-	-	b	(Dialysed
-	8	16	16	8	8	16	8	16	16	c	F_7)
-	16	16	8	-	8	8	-	32	16	e	
16	8	32	32	8	16	16	8	32	16	f	
-	-	?	-	-	-	-	-	-	32	a	E
8	AC 64	128	32	32	16	32	32	8	8	c	(Dialysed F_7 in
-	AC 32	8	-	-	-	-	≠			d	Freunds Complete Adjuvant)
16	-	-	8	-	-	-	16	-	16	e	
16	32	32	128	64	16	16	8	-	8	f	

- = negative or <4 ≠ = animal died AC = Anti-complementary

Table 27

Effect of heating sera at 56°C. for 30 mins. on their activity in complement fixation test against F_7

Rat		0	4	7	10	14	18	22	28	32	38
Aa	U	-	64	-	16	32	32	32	-	-	-
(AC)	H	NT	32	NT	4	8	4	-	NT	NT	NT
Ab	U	-	64	64	32	64	128	-	-	-	32
(AC)	H	NT	32	16	-	NT	4	NT	NT	NT	8
Ac	U	32	64	256	128	128	256	64	64	64	≠
	H	8	64	16	16	32	32	8	-	NT	≠
Af	U	16	64	32	64	32	32	32	128	32	≠
(AC)	H	-	32	8	8	-	-	-	4	-	≠
Ba	U	16	512	256		32	16	64	32	16	≠
	H	-	256	64	16	8	4	-	8	NT	
Bb	U	16	-	-	32	32	64	32	32	32	32
	H	-	NT	NT	-	-	32	8	16	8	-
							AC	AC			
Bf	U	-	512	64	64	128	64	32	-	-	32
(AC)	H	NT	16	-	16	32	32	-			
Ce	U	-	128	16	32	-	32				
	H	NT	16	NT	NT	NT	-				

- = negative or <4

≠ = animal died

AC = Anti-complementary

Table 28

Complement Fixation Titres of Sera against autologous and homologous preparations of F_7

Animal	Homologous	Autologous
Aa D_0	-	-
4	16	32
14	16	8
18	16	8
Ad D_0	16	16
4	64	32
32	32	32
38	8	16
Bc D_0	-	-
18	64	32
22	64	128
28	64	64
Bf D_0	-	-
4	128	256
7	32	32
10	64	32
14	32	64

- = negative or less than 1/4

The top half of the supernate was removed and tested in the complement fixation test against 4 known positive sera. The results are shown in Table 29 and it can be seen that recentrifugation has no gross effect on the antigenic activity of the fractions. A preparation of F_7 was fractionated by ultracentrifugation at 35,000 RPM for 16 hours in a sucrose density gradients. Nine fractions were taken. Each fraction was diluted out and tested in the complement fixation test against a constant dilution of a known positive sera. The results showed that the activity was distributed equally throughout the fractions.

The possibility arose that the soluble antigen occurred as a result of breakdown of sedimentable tissue components by the in vitro homogenisation and fractionation procedures. Further studies were carried out to test this hypothesis.

Normal rats were bled and each injected with 0.5 ml. F_7. Four days later they were bled again. The sera were taken and stored at -25°C. until required for testing. Liver fractions were diluted out and tested in the complement fixation test against 1/8 dilutions of these sera. The results (Table 30) showed that the injection of F_7 resulted in increased complement fixing activity against all the fractions rather than an increase in activity against F_7 alone.

Four rats were injected with F_7 and bled 4 days later. The sera were taken and divided into aliquots. One aliquot (untreated)

Table 29

Effect of recentrifugation on the antigenic activity of F_7 and dialysed F_7

Serum	Serum Titres in complement fixation test			
	F_7	F_7 centrifuged for 1.8×10^7 g mins	Dialysed F_7	Dialysed F_7 centrifuged for 1.8×10^7 g mins.
1	16	16	16	32
2	16	16	32	32
3	128	64	64	32
4	64	32		

Table 30

Antigen dilution tests with normal sera and sera taken 4 days after the injection F_7 against liver fractions.

Figures represent highest antigen dilution giving positive complement fixation test

Serum	WLH	F_1	F_2	F_3	F_4	F_5	F_6	F_7
Ab 0	NT	160	240	240	80	80	40	<5
Ab 4	NT	320	320	320	160	160	120	80
Aa 0	NT	160	240	240	160	40	10	< 5
Aa 4	NT	240	480	480	320	120	80	120
1 D_0	1280	320	320	640	160	160	80	10
1 D_4	5000	640	320	1280	320	320	160	160
2 D_0	640	160	160	320	80	40	10	< 10
2 D_4	1280	160	160	320	80	40	40	40

NT = Not tested

was stored at -25°C. and the other absorbed with F_3. Each aliquot (0.2 ml.) was mixed with about 100 mg. (wet weight) washed F_3 and allowed to stand at room temperature for 30 minutes and overnight at 4°C. The mixtures were centrifuged for 9×10^5 g minutes and the supernates removed. The absorbed aliquots and the untreated aliquots were then tested in the complement fixation test against F_3 and F_7. The results (Table 31) showed that the removal of anti-F_7 activity from the sera, by absorption with F_3, paralleled the removal of anti-F_3 activity.

Influence of maternal antibody on the induction of tolerance

There is evidence that the induction of tolerance by neonatal exposure to a particular antigen may be prevented by the simultaneous administration of specific anti-serum. This led to a consideration of the possibility that transfer of specific maternal antibody to her offspring may occur and interfere with the induction of tolerance to the corresponding antigen. In rats some maternal antibody is known to pass via the yolk sac to the foetus although the majority is transferred in the colostrum (Good and Papermaster, 1964). The following experiment was designed to provide evidence for or against this hypothesis.

Female rats were given injections of 5 mg. alum precipited bovine serum albumin (BSA) at 8 weeks, 30 days and 18 days before the birth of litters. Their litters were injected

Table 31

Complement Fixing Activity against F_3 and F_7 of rat sera, before and after absorption with F_3

Serum	F_3	F_7	Anticomplementary Control
A Untreated	64	32	negative
Absorbed	16	8	
B Untreated	64	32	negative
Absorbed	<4	<4	
C Untreated	128	32	negative
Absorbed	16	8	
D Untreated	512	128	negative
Absorbed	64	32	

within 24 hours of birth with either 3 mg. or 5 mg. alum precipitated BSA. Littermate controls were injected with alum alone. Contemprarious controls were set up in which litters from untreated rats were injected with 3 mg. or 5 mg. alum precipitated BSA. The litters were challenged at 5 weeks after birth with 5 mg. alum precipitated BSA and bled 12 days later. The sera were tested for their ability to bind BSA by the ammonium sulphate precipitation technique of Farr (Materials & Methods p. 42). The results (Table 32) gave no indication that maternal anti-BSA antibody was able to interfere with the induction of tolerance to BSA in their litters. The sera of the primed mothers was tested, and found to contain high levels of anti-BSA antibody.

Maturity of anti-tissue antibody forming cells

If the immunological system matures early in ontogenesis to the tissue antigens which react with the anti-tissue auto-antibodies described in this thesis, then rats should respond immunologically to these antigens early in ontogenesis. An attempt was made to test this hypothesis by measuring the ability of rats to respond to tissue breakdown products, induced by toxic liver damage, shortly after birth.

Litters of rats of various ages were injected subcutaneously with carbon tetrachloride. Each rat received .005 ml. CCl_4 diluted in 0.045 ml. liquid paraffin/10 gm body weight or 0.05 ml.

Table 32

Antigen binding capacity at 0.01 ug N BSA*			
	Neonatal Treatment		
Mother	3mg BSA	0.5mg BSA	alum alone
Primed	< .005		
	< .005		
Primed		< .005	.15
		< .005	.30
		.31	.24
		.15	.25
		.07	
		< .005	
Normal	.006	.017	
	< .005	< .005	
	< .005	.13	
	< .005	.016	
		.58	
Normal	.017	< .005	
	< .005	.017	
	< .005	< .005	
	< .005		

liquid paraffin/10 gm body weight. Four days after injection they were bled and the sera collected and stored at -25°C. until required for testing. The sera were tested in the complement fixation test against a mitochondrial preparation of rat liver (F_3). The results are recorded in Table 33. It can be seen that in every one of the eight groups the rats injected with CCl_4 have, on average, higher levels of complement fixing anti-mitochrial antibody than the controls. This in itself shows that there is a significant ($p < 0.01$) difference between the experimental and control groups and it seems unnecessary, therefore, to resort to more sophisticated statistical analysis.

Table 33

Reciprocal C.F. Titres

	Test 0.005 CCl_4 in 0.045 liquid paraffin		Control 0.05 liquid paraffin	
	Unheated	Heated	Unheated	Heated
Litter 1	6	-	12	-
injected D_3	24	-	24	-
bled D_7	24	-	12	-
	24	-	12	-
	24	-		
Litter 2	48	-	24	-
injected D_3	96	-	24	-
bled D_7	24	-	48	-
	48	-	24	-
	192	3		
Litter 3	24	3	12	-
injected D_4	48	-	24	-
bled D_8	24	6	3	-
	12	6		
Litter 4	192	3	48	-
injected D_5	96	-	-	-
bled D_9	96	-	24	-
	96	-	24	-
	24	-	12	-

Table 33

Reciprocal C.F. Titres

	Test 0.005 CCl_4 in 0.045 liquid paraffin		Control 0.05 liquid paraffin	
	Unheated	Heated	Unheated	Heated
Litter 5	48	3	12	-
injected D_6	48	-	12	-
bled D_{10}	24	3	6	-
Litter 6	48	3	48	-
injected D_7	24	-	48	-
bled D_{11}	48	-	24	-
	96	-	12	-
	192	-	48	-
	48	-	24	-
Litter 7	24	3	24	-
injected D_8	48	3	12	-
bled D_{12}	24	-	12	-
	24	-		
	48	-		
Litter 8	48	-	24	-
injected D_9	12	3	6	-
bled D_{13}	48	6		

- = <3

D I S C U S S I O N

DISCUSSION

The primary purpose of this work was to determine whether anti-tissue autoantibodies could be detected in "normal" rat sera. The results have shown that most "normal" rat sera contain a factor reactive with tissue constituents. The factor was considered to be an autoantibody on the basis of observations of its behaviour in experimental situations.

"Normal" unheated rat sera were found to fix guinea-pig complement in the presence of tissue homogenates (Section 1). There are, however, a number of situations not associated with antigen-antibody union in which complement is known to be activated e.g. heat denaturation and aggregation of globulin (Christian, 1960; Ishizaka, Ishizaka & Borsos, 1961) and standing sera at room temperature (Zinsser & Johnson, 1911). Such anticomplementary effects were excluded in this work by serum controls containing twice as much serum as the test. Beal (1963) contended that the ability of certain "abnormal" human sera to fix complement in the presence of homologous liver extract should be attributed to the aggregation of "abnormal 7S globulins with normal γ globulin-like proteins attached to the tissue, rather than to antigen-antibody interaction. This mechanism seems unlikely to account for the results obtained here since "normal" rat sera were found to fix complement in the presence of mitochondrial preparations of rat liver (F3). The isolated mitochondria themselves were shown to be free from contaminating serum proteins as judged by the failure to detect antibodies against rat serum components in the sera of rabbits

immunized with F_3. These findings suggested that complement was fixed as a result of an interaction between tissue antigens and antibody present in normal sera.

The specificity of this reaction was shown by the results of absorption studies. The anti-mitochondrial activity was found to be completely removed by treatment of "normal" sera with washed F_3. Similar treatment of "normal" sera with sheep erythrocytes or rabbit anti-bovine serum albumin (BSA)-BSA complexes was found to have no effect on the anti-F_3 activity. In addition it was noted that normal rat sera did not give positive reactions in the haemolytic complement fixation test with antigens such as BSA, brucellin and fungal antigens. The observation that normal rat sera were able to fix complement with autologous liver homogenates showed the autoreactive nature of the factor.

Testing fractions of rat sera obtained by Sephadex chromatography and zone ultracentrifugation in sucrose density gradients, showed that the anti-tissue complement fixing activity was associated with the high molecular weight immunoglobulins (IgM) (Section 3). Attempts to detect anti-tissue activity in IgM fractions freed from contaminating $\alpha 2$ macroglobulins by pevikon block electrophoresis were made. They failed due to the high anti-complementary activity of the fractions. However, there is no evidence to suggest that $\alpha 2$ macroglobulins participate in antibody assays. Moreover, Weir (1964,a) has demonstrated that the anti-liver activity of the auto-antibody induced by toxic liver

damage is in IgM fractions free from contaminating $\alpha 2$ macroglobulins. Thus the detection of anti-tissue activity in IgM fractions of "normal" rat sera, using a variety of immunological techniques, provides evidence for the antibody nature of the factor. In this work the reaction between IgM fractions and tissue antigens was demonstrated by complement fixation, passive haemagglutination (Section 3) and uptake of IgM by subcellular fractions (Section 6). Other workers (Weir et al, 1966) have demonstrated the binding of IgM (prepared from "normal" rat sera) using fluorscein conjugated anti-rat serum. Similarly they detected the fixation of added guinea-pig complement by means of fluorescein labelled anti-guinea pig globulin.

The finding that a purified mitochondrial preparation of rat liver (F_3) selectively takes up immunoglobulins and complement components from normal rat sera (Section 6) provides evidence in support of the proposition that the serum factor reactive with tissue constituents is an antibody. Rabbits injected with F_3 coated with normal rat sera (NRS) were found to produce antibodies against rat immunoglobulins and complement components but not antibodies against other rat serum components e.g. albumin (Figs. 13, 14 & 15). Using immunization and immunoelectrophoretic procedures similar to those described here (Materials & Methods p.49), other workers have noted the selective uptake of immunoglobulins and complement components from anti-sera by the corresponding antigens. Arnason, de Vaux St-Cyr & Relyveld (1964) showed that IgM,

IgG and IgA were taken up from rat anti-diptheria toxin and rat anti-bovine serum albumin (BSA) by diptheria toxin and BSA respectively. Similar findings were reported by Beernink & Steward (1967) using E.coli. and both normal guinea pig sera and guinea pig anti-E.coli. sera. They demonstrated the binding of at least 7 guinea pig serum components on E.coli. including IgG (γ_1 and γ_2), IgM and three complement components. These observations suggest that the uptake of immunoglobulins and complement components from normal rat sera by F_3 was a result of antigen-antibody combination rather than a result of some non-specific absorptive effect.

Binaghi, Oriol & Boussac-Aron (1967) have shown that guinea pigs injected with heterologous immunoglobulins produced antibodies against the class specific F_c antigenic determinants only. Similarly in this work it was found that a guinea pig injected with F_3 coated with a purified rat IgM preparation produced antibodies only against rat IgM (Fig. 19) It was also found that a guinea pig injected with F_3 coated with "normal" rat serum produced antibodies against IgM (Fig. 17). In contrast, rabbits injected with F_3 coated with a purified rat IgM preparation produced antibodies against IgM, IgG and IgA. These findings raise the possibility that the uptake of IgG and IgA on F_3, shown in Figs. 13 & 14, is an apparent effect due to rabbits producing antibodies against the light chain determinants of IgM which would thus precipitate IgG and IgA. However, it was found that

IgG from two out of three purified rat IgG preparations was taken up on F_3. Rabbits injected with F_3 coated with purified rat IgG produced antibodies against rat IgG (Fig. 18). It may be that this uptake is nonspecific arising from changes induced in the IgG on purification, but it cannot be excluded that it is a result of an antigen-antibody reaction between an IgG anti-tissue auto-antibody and F_3.

Further work supporting the conclusion that a specific reaction took place between F_3 and an antibody in normal rat sera is provided by the work of Weir & Suckling (1968). Using the immuno-cytoadherence technique (Biozzi et al, 1966) they showed that "normal" rat spleen cells form more rosettes with mitochondrial (F_3) coated sheep erythrocytes than with sheep erythrocytes alone. After the injection of CCl_4 there was a marked increase in the number of rosette forming cells with mitochondrial coated sheep erythrocytes. There is evidence that this technique provides a direct measure of antibody production by spleen cells (Cunningham, Smith & Mercer, 1966; Biozzi et al, 1968) although the results obtained may be only "semi-quantitative" (Zaalberg, Van der Meul & Van Twisk, 1968).

Antibodies were classically defined as a group of serum proteins with the capacity to combine specifically with inciting substances (Landsteiner, 1945). They are now known to be a heterogeneous group of serum proteins with respect to structural and biological properties. For instance the higher molecular

weight IgM antibodies appear to be more efficient at complement fixation (Humphrey & Dourmashkin, 1965) and haemagglutination (Greenbury, Moore & Nunn, 1963) than the lower molecular weight IgG. On the other hand IgM antibodies do not take part in skin tests of the passive cutaneous anaphylaxis type (Brocklehurst, 1968). Similarly some high affinity IgG antibodies bind soluble protein antigens but do not precipitate them (Farr, 1958). Clearly then a particular type of antibody may not be effective in all serological tests. In the present state of our knowledge a serum factor such as that described here can only be classified as an antibody by examining its properties and comparing them with those of a particular class of immunoglobulin. The anti-tissue serum factor has been shown to possess features associated with antibodies of the IgM type e.g. the specificity, the ability to fix complement and agglutinate tissue antigen coated erythrocytes, the sedimentation and gel filtration characteristics and the presence of rosette forming cells in the spleen. There is thus a considerable weight of evidence in favour of the complement fixing anti-tissue factor being an IgM autoantibody.

It has not been unequivocally demonstrated that anti-tissue antibodies of the IgG type occur in "normal" rat sera. It was found that a rabbit and a guinea pig injected with fraction 3 (F_3) coated with a purified preparation of IgG from "normal" rat sera, produced antibodies against rat IgG. This suggests that, if a suitable test is used, it may be possible to detect anti-tissue

antibodies of the IgG type in "normal" rat sera. In this respect it is interesting that IgG anti-tissue autoantibodies were not detected by complement fixation in the sera of rats or rabbits, following tissue damage (Weir, 1963; Elson, 1965, Section 3; Asherson & Rose, 1963). In contrast Shulman et al (1968), using passive haemagglutination were able to detect low levels of IgG anti-tissue autoantibody about 16 days after cryosurgical tissue damage in rabbits and Weir & Elson (1968) have found that haemagglutination is a more suitable test for detecting rat IgG erythrocyte antibodies than complement lysis. However, in this work no IgG anti-tissue autoantibodies were detected in the sera of "normal" rats by passive haemagglutination.

Response to Tissue Damage

Sub-cellular fractions, prepared by differential centrifugation of tissue homogenates, were tested against normal rat serum using the haemolytic complement fixation test. The results (Section 4) showed that the complement fixing activity in normal rat sera, like that of the heat stable autoantibody induced by tissue damage (Pinckard & Weir, 1966), was directed primarily against the mitochondrial fractions. This observation suggests that the heat stable autoantibody is an "enhanced" level of the antibody present in "normal" rat sera.

The complement fixing activity of the anti-tissue antibody present in "normal" rat sera was found to be reduced or removed

by heating the sera at 56°C. for 30 minutes prior to testing (Section 2). However, a considerable proportion of the complement fixing activity of the anti-tissue antibody induced by toxic liver damage was found to be resistant to heating at 56°C. for 30 minutes (Section 2). These findings led to an investigation of the heat lability of other rat antibodies (Section 5). Studies on the primary binding and complement fixing activity of rat anti-bovine serum albumin (BSA) sera, before and after heating at 56°C. for 30 minutes, showed that the primary binding of such anti-sera (or IgM fractions) was not affected by heating. In contrast the complement fixing activity of these sera was found to be markedly reduced by the heating procedure. In addition Weir (Weir & Elson, 1968) found that although the agglutinating activity of rat anti-sheep erythrocyte sera was not affected by heating the sera at 56°C. prior to testing, the lytic activity was partly destroyed. The lytic and agglutinating activity of the anti-sheep erythrocyte sera was found to be associated with the IgM containing fraction on zone ultracentrifugation. These observations, together with the known role of the Fc fragment in complement fixation (Ishizaka, 1963) indicate that heating at 56°C. affects the Fc portion of the rat IgM molecule rather than its antigen binding Fab fragment (see also Fig. 12). Similarly, the possibility arose that the heat induced reduction in complement fixing activity of the IgM anti-tissue antibody may be a reflection of changes in the Fc portion of the molecule rather than the antigen

binding Fab fragment. This possibility was substantiated by the finding that F_3 was still able to selectively take up immunoglobulins from heated (56°C. for 30 minutes) "normal" rat sera as judged by the presence of anti-rat immunoglobulin antibodies in the serum of a rabbit immunized with F_3 treated with heated "normal" rat sera (Section 6).

The induction of tissue damage in rats, following the injection of CCl_4, was shown to result in an increase in the complement fixing activity of IgM anti-tissue autoantibodies (Sections 2 and 3). There is some evidence that the complement fixation test measures both the quality and quantity of antibodies. Wallace, Osler & Mayer (1950) showed that anti-sera from rabbits which had received two courses of inoculations fixed more complement/unit antibody weight than anti-sera from rabbits given only one course of immunisation. However, it is not clear whether the increase in overall complement fixing activity induced by tissue damage can be partially attributed to an increase in the affinity of the antibody produced. It has been shown that the average association constant of "naturally occurring" anti-human blood group B antibody was of the order of 10^6 litres moles^{-1} (Wurmser & Filitti-Wurmser, 1937) whereas the average association constant of an "immune" IgM anti-human blood group B antibody was estimated to be about 10^8 litres moles^{-1} by Economidou, Hughes-Jones & Gardner (1967). Changes in affinity of this magnitude for anti-dinitrophenylated bovine γ globulin antibodies have been

shown to occur over a matter of weeks rather than days (Eisen & Siskind, 1964). These results together with the apparent brevity of the response to tissue damage suggest that changes in affinity of the anti-tissue antibody are likely to be relatively small. However, the results (Section 2) showed that there was a considerable increase in the proportion of heat stable complement fixing antibody after tissue damage. In view of the results discussed above it seems likely that this increase in heat stability of the complement fixing activity reflects changes in the Fc portion of the molecule rather than the antigen binding Fab fragment. Thus the immunological response to tissue damage appears to be an increase in the production of complement fixing IgM anti-tissue autoantibodies. The increase in stability of this antibody seems likely to be due to changes in the Fc portion of the antibody molecule although whether these changes are associated with changes in the antigen binding Fab fragment is not clear. However, it may be that the changes in the stability of the anti-tissue antibody are of considerable physiological importance.

The response to tissue damage appears to have the features of a "secondary" response in that it is detected shortly after the release of liver antigens (Weir, 1963, 1966, Section 2). However, the antibody detected in this response has been shown to be IgM in type (Weir 1964,a, Section 3) and there is considerable controversy centred around the question of whether or not there is a mechanism of immunological memory for antibodies of the IgM type. In two

intensively investigated systems, namely the response of guinea pigs to phage antigen (Uhr & Finkelstein, 1963) and of rabbits to poliomyelitis virus (Svehag & Mandel, 1964), the injection of small doses of antigen resulted in IgM formation with no detectable IgG. A similar dose given some weeks later resulted in an almost identical response suggesting that there was no immunological memory for IgM. Uhr & Finkelstein also showed that the anti-phage IgM response could be prolonged by a second injection of phage 5-9 days after the primary injection. Antibody levels were found to rise within 24 hours and they took this to indicate the synthesis of antibody by the same population of cells which had previously produced IgM. These observations suggest that the higher levels of anti-tissue antibody found after tissue damage are produced by cells already involved in the synthesis of anti-tissue antibody and/or by the stimulation of virgin cells. However, Nossal, Austin & Ada (1965) showed that an enhanced IgM secondary response could be obtained in certain circumstances. They followed the IgM response to the primary and secondary (6 weeks later) injection of the flagella antigen of Salmonella adelaide in rats. Higher levels of anti-flagella IgM were only detected in the secondary response if the challenge dose was equal to or greater than the primary dose. This finding led Nossal to suggest that there was some form of immunological memory for IgM, perhaps mediated by small lymphocytes of the type shown to carry immunological memory to phage ϕ X 174 in rats (Gowans & Uhr, 1966).

If this is so then it seems reasonable to postulate that the release of large amounts of tissue breakdown products may stimulate the appropriate memory cells to synthesise anti-tissue antibody. Moreover, the increase in proportion of heat stable anti-tissue antibody can be explained by supposing that the memory cells synthesise predominantly heat stable antibody. If this is not the case and there is no immunological memory cell for IgM antibodies, then there must be some form of selective synthesis of heat stable anti-tissue antibody following tissue damage.

Thus two related problems have emerged from this discussion and remain to be solved:-

(i) Whether there is a change in affinity of anti-tissue antibody in response to tissue damage comparable to those found in immune responses to other antigens. Furthermore, if there is an increase in affinity, whether this change is associated with an increase in stability of the antibody.

(ii) Whether there is a memory cell for anti-tissue antibodies of the IgM type and if not how the selective synthesis of heat stable anti-tissue antibody occurs.

Origin of Anti-tissue Antibodies

The finding of anti-tissue antibodies in the sera of "normal" rats raises the question of how these antibodies arise. Hook, Toussaint, Simonton & Muschel (1966) traced the level of naturally

occurring antibodies to a variety of antigens from birth to maturity in rabbits. They were able to detect natural antibodies to Shigella dysenteriae by a bacteriocidal test, to coliphage T_2 by a phage assay and to homologous liver homogenate by a complement fixation test involving the use of heated 56°C. for 30 minutes) sera. This led them to postulate that these antibodies arise at the same time as a result of normal physiological maturation and that globulins react with certain antigens because of chance chemical correspondence with certain specific combining groups i.e. they do not necessarily arise as a result of immunization. However, it is well known that there is a considerable variation in the sensitivity of serological tests (see Marrack, 1963) and particularly those measuring secondary and tertiary effects of the primary antigen-antibody reaction (Weir, 1967). Additionally in this work it was found that the complement fixing activity of rat anti-sera was markedly reduced by heating at 56°C. and heat labile complement fixing anti-tissue antibodies were detected in the sera of rats within 24 hours of birth. These observations suggest that the failure to detect anti-liver antibodies before 40 days in rabbits (Hook et al, 1966) was merely due to the insensitiveness of the test employed and/or the heat lability of the complement fixing activity of the anti-liver antibody. Moreover, in view of the work of Sterzl and his co-workers (Sterzl & Silverstein, 1967) in which it was found that natural antibodies could not be detected

in the sera of germ-free piglets fed on non-antigenic diets, it is likely that antibodies reach detectable levels only in response to some antigenic stimulation.

There is evidence that certain bacteria share antigenic determinants with mammalian tissue components. For example the finding that rabbit anti-sera to T 12 nephritogenic streptococci was nephrotoxic to rats was taken to indicate a sharing of antigens between the streptococci and rat glomerulus by Markowitz, Armstrong & Kushner (1960). Similarly, Kaplan (1963) demonstrated cross reacting antigens between certain group A streptococci and human heart. Rabbit anti-seraagainst a cell wall preparation of the streptococci gave immunofluorescent reactions with human heart. Moreover, precipitating antibodies against the streptococcal cell wall preparation in the sera of patients with streptococcal infections and rheumatic heart disease were found to be removed by absorption of the patients sera with human heart preparations (Kaplan & Svec, 1964). The work of Perlmann, Hammerstrolm, Lagercrantz and Gustafsson has provided evidence of antigenic sharing between rat colon and lipopolysaccharide fraction E. coli 014. The lipopolysaccharide fraction was found to give a good inhibition of the haemagglutination of germ-free rat colon sensitised erythrocytes by the serum from a patient with ulcertative olitis. Furthermore, anti-colon autoantibodies have been detected by the indirect immunofluorescent technique in the sera of rabbit following the injection of certain dead bacteria in Freunds complete

adjuvant (Asherson & Holborow, 1966). These observations, together with the finding that autoantibody production in the rabbit appears to be more readily induced by heterophile (Asherson & Dumonde, 1962; Holborow, Asherson & Wigley, 1963) or chemically altered (Weigle et al, 1967) tissue antigens than by homologous tissue preparations led to the suggestion that some anti-tissue antibodies may arise as a result of cross immunization by bacterial antigens (Asherson, 1966; Glynn & Holborow, 1965).

In this work it was shown that rats responded to tissue damage by producing complement fixing anti-tissue antibodies which appear to be an "enhanced level" of these found in normal rat sera. Anti-liver antibodies were detected in the sera of neonatal rats. The titres of this antibody rose rapidly over the first 2 - 3 weeks of life reaching adult levels by 3 - 6 weeks and it was found that higher titres of this antibody could be induced during this period by toxic liver damage. These findings showed that ratswere able to produce anti-liver antibody in response to tissue damage from early in life. This suggests that the anti-tissue antibodies found in normal rat sera arise as a result of stimulation by tissue breakdown products rather than as a result of cross immunisation with bacterial or other antigens. In support of this suggestion is the demonstration by Arnason, Salomon & Grabar (1964) that the immunological response to liver damage was similar in both normal and germ-free mice. Elevated levels of anti-tissue antibody were found in both normal and germ-free mice following

the injection of CCl_4. Moreover, a number of workers have described conditions in which tissue damage has led to auto-antibody production. For instance Irvine (1964) found rises in the titres of complement fixing anti-thyroid antibodies in thyrotoxic patients following treatment with radio-iodine. Similar rises in titres of anti-skin autoantibodies have been found in the sera of burned patients (Pavkova, 1962). Using the colloidon particle agglutination method antibodies were detected on the third day after injury and fell at the time of intensive clinical healing. Shulman and his co-workers (Shulman, Yantarno & Bronson, 1967; Shulman, Brandt & Yantarno, 1968 a) showed that destructive freezing in the coagulating gland and seminal vesicle of the rabbit stimulated the production of an organ specific autoantibody. The autoantibody response was detected by passive haemagglutination and was of short duration (7 - 10 days). The autoantibody was found to be predominantly of the IgM type. Finally, it seems pertinent to mention a case in which tissue damage associated with bacterial infection, resulted in autoantibody formation. Asherson & Rose (1963) showed that the sera of rabbits experimentally infected with Eimera stiedae during the first 7 weeks of life had significantly higher titres of IgM complement fixing anti-kidney autoantibody than littermate controls.

Anti-tissue Antibodies and Inflammation

Polymorph migration

In this work it was shown that the complement fixing reaction between normal rat serum and tissue homogenates was mediated by a heat labile IgM autoantibody and the tissue antigen with which it reacts. Mixing normal rat serum with a mitochondrial preparation of rat liver (F_3) was found to induce the migration of polymorphonuclear leucocytes (PMN) as judged by Boyden's chamber technique (Section 7 Table 15). The possible role of complement components in this reaction is indicated by the fact that heating the serum before mixing with the mitochondrial fraction inhibited the development of the chemotactic factor which was shown itself to be heat stable (Table 16). These findings are similar to those reported by Boyden (1962), using human serum albumin (HSA) rabbit anti-HSA mixtures. It was he who originally suggested that the heat stable chemotactic factor is produced as a consequence of an antigen-antibody reaction activating heat labile complement components. Keller & Sorkin's (1965) finding that the haemolytic complement fixing activity of various γ-globulin preparations did not correlate with their ability to induce chemotaxis, appears contradictory to Boyden's suggestion. Keller & Sorkin found that treatment of heat aggregated human gamma globulin at pH 4.0 eliminates its ability to fix guinea pig complement although its capacity to exert a chemotactic effect on PMN, in the presence of fresh serum,

remained unimpaired. However, the work of Ward and his co-workers (Ward, Cochrane & Müller-Eberhard, 1965, 1966; Müller-Eberhard, 1968) confirmed Boyden's contention by identifying the principal chemotactic factor as a tri-molar complex of C'5, C'6 and C'7. They found that sheep erythrocytes sensitised with rabbit amboceptor were not chemotactic for PMN in the presence of purified preparations of the first four components of guinea pig complement. However, the mixture became chemotactic for PMN on the addition of C'5, C'6 and C'7 prepared by eluting the euglobulin fraction of fresh guinea pig serum from TEAE cellulose. In addition fractions of rabbit serum activated by treatment with BSA-antiBSA complexes were prepared by Pevikon block electrophoresis and tested for chemotactic activity. It was found that only those fractions containing both C'5 and C'6 were active. These results were interpreted as showing that the interaction of the first four components of complement leads to the activation of the C'5, C'6 and C'7 components which are then transformed in a chemotactically active complex. It may be that the failure of Keller & Sorkin to relate haemolytic complement fixation with the formation of the chemotactic factor can be explained by the kinetics of its formation (see review by Müller-Eberhard, 1968). Thus Ward, Cochrane and Müller-Eberhard (1966) found that the ratio of the components (C'5, C'6 and C'7) was critical for the full expression of chemotactic activity. Maximal chemotactic activity was associated with a relative excess of a C'5 fraction. Similarly, Boyden (1962)

found that PMN migration was maximal if the antigen and antibody were mixed at equivalence and this was also found to be a feature of the system described in this thesis. Further support for an activated component of complement being the principal chemotactic agent responsible for PMN migration has come from studies on rabbits with a recessively inherited absence of the C'6 component of complement. Such rabbits were found to exhibit minimal direct passive Arthus reactions to the intradermal injection of bovine serum albumin and human γ globulin as compared with heterozygotes of the same strain. The reactivity of the C'6 deficient rabbits was found to be considerably increased by mixing purified C'6 with the test antigen. This impairment of the Arthus reaction in C'6 deficient rabbits was shown to be related to an impairment in the polymorph migration to the site of injection. Thus, the failure of an antigen-antibody interaction to induce PMN migration in these rabbits was associated with their inability to produce the C'5, C'6 and C'7 chemotactic agent (Rother, Rother & Schindera, 1964; Rother, Rother, Müller-Eberhard & Nilsson, 1966; Biro, 1966).

Hurley (1963, 1964), using Boyden's chamber technique and direct observation of rat skin sections, demonstrated that the migration of PMN towards tissue homogenates mixed with normal rat serum was dependant on a chemotactic factor produced by a serum-tissue homogenate interaction. The results described in this thesis thus extend Hurley's work by relating this phenomenon to a

reaction between the complement fixing IgM antibody in normal rat serum and the corresponding tissue antigen. In support of the IgM anti-tissue antibody in normal rat serum being that concerned in bringing about the chemotactic effect on combination with F_3, was the observation that F_3 treated with normal rat serum takes up various immunoglobulins including the IgM anti-tissue antibody together with the complement components it has activated (Section 6). As discussed previously it seems likely that this uptake is a result of an antigen-antibody reaction between F_3 and the anti-tissue antibody.

Clearance of tissue breakdown products

It has been proposed that autoantibodies in normal serum have an essential physiological role in the removal of tissue breakdown products (Grabar, 1957, 1959; Boyden, 1963, 1964; see Introduction p. 29). The results reported here support this hypothesis by suggesting that an initial step in the clearance of tissue breakdown products is the binding of complement fixing anti-tissue antibody which activates the chemotactic factor causing attraction of PMN. However, it is not clear if PMN are able to phagocytose sub-cellular tissue breakdown products. Vaughan (1965 a,b) found that rabbit PMN did not readily phagocytose effete autologous lymphocytes even in the presence of 'normal' serum, although they exhibited considerable phagocytic activity towards other test particles such as carbon, carmine and staphylococci. In contrast,

macrophages were found to exhibit considerable phagocytic activity towards effete autologous lymphocytes. These findings suggest that PMN are not important in the phagocytosis of effete material. It seems likely, therefore, that PMN have some other role in the clearance of tissue breakdown products.

It is well established that serum antibodies facilitate the phagocytosis of foreign material by macrophages (Perkins & Leonard, 1963; Stiffel et al, 1964). Similarly, autoantibodies have been implicated in the phagocytosis of homologous tissue material by peritoneal macrophages (Stuart, 1967a). However, there is some controversy as to whether the uptake of autologous effete material is affected by opsonins. Vaughan, (1965 a,b) found that the uptake of effete autologous lymphocytes by rabbit macrophages was not markedly increased by the presence of 'normal' serum. In contrast Jenkins & Karthigasu (1962) showed that the uptake of aged autologous erythrocytes by the isolated perfused liver was dependant on the presence of an 'auto-opsonin'. Fresh autologous or washed aged erythrocytes were not taken up from the perfusate, whereas aged autologous erythrocytes treated with autologous serum were removed. Macrophages isolated from different parts of the body are known to be heterogeneous with respect to their phagocytic discrimination (see Gesner & Howard, 1967 and Stuart, 1967b). Thus it may be that some macrophages are not capable of phagocytosing effete autologous material without the participation of opsonins. In this work <u>in vitro</u> attempts to measure the uptake

of F_3 by peritoneal macrophages failed (Section 7) although it was shown that $I^{131}F_3$ injected intracardially in rats was taken up predominantly by the liver. Clearly it is necessary to determine whether or not auto-opsonins play any part in the uptake of tissue breakdown products by phagocytes.

PMN and macrophages have been found to migrate concurrently, with no selective migration of cell type, from venules following the injection of various macromolecular substances into the rat abdominal wall (Paz & Spector, 1962). This suggested that the same stimulus may be responsible for both PMN and mononuclear cell migration (see Page, 1964). In this work peritonealcells (macrophages) were not found to be attracted by serum-tissue interactions as measured by Boyden's chamber technique (Table 20) and similarly Boyden (1963) was unable to detect migration of macrophages towards other antigen-antibody interactions. Volkman & Gowans (1965 a,b) have utilised tritiated thymidine, which is incorporated into the DNA of rapidly dividing cells, to show that macrophages in foci of sterile acute inflammation in rats are derived from blood monocytes which are in turn derived from rapidly dividing cell lines in the bone marrow. Monocytes, furthermore, are known to transform into large macrophages at the site of inflammation (Florey& Jenning, 1962). The finding that macrophages are not attracted by serum-tissue interaction must be treated with caution since the peritoneal cells used as a source of macrophages may not contain mononuclear cells in a state in which they are capable of responding to chemotactic

stimuli. However, peritoneal macrophages were found to be attracted to particulate effete material such as cell membrane fractions of rat liver (F_1 and F_2) and to particulate fraction of ultrasonically disintegrated PMN (Tables 21 and 22). In contrast they were not attracted to light mitochondrial and microsomal fractions of rat liver (F_3, F_4, F_5, F_6, and F_7) or a supernatant fraction of PMN. Golub & Spitznagel (1964) have shown that the intradermal injection into rabbits of a lysosomal fraction of homologous PMN, prepared by centrifugation of disintegrated PMN at 8000G, causes increased vascular permeability and attraction of PMN and mononuclear cells. Fractions prepared by centrifugation of disintegrated PMN at 200G and over 8000G were found to be inactive. These observations raise the possibility that PMN, attracted to the site of tissue damage as a result of tissue antigen-anti-tissue autoantibody interaction, may facilitate clearance of tissue breakdown products by other phagocytes. One possibility is that they may release enzymes at the site of tissue injury which activate other pharmacologically active agents. For instance, Ward (1967) has described a factor produced by the action of proteolytic enzymes on C'3 which was found to be chemotactic for PMN and to increase vascular permeability on injection into rat skin. Another possibility is that PMN may liberate pharmacologically active agents which they are known to contain e.g. histamine (Osler, Lichenstein & Levy, 1968), at the site of tissue damage. A final possibility is that PMN may in some way affect the transformation of mononuclear cells to macro-

phages at the site of tissue damage (see Elves, 1967). Evidence in favour of this possibility comes from studies of the inflammatory response of a patient with cyclic neutropenia. Good and Page (1958) found that in the neutropenic phase, the cellular response to sterile skin injury was characterised by a dearth of macrophages.

The problem remains as to what the stimulus is for the migration of monunuclear cells from the small venules at the site of tissue injury. It has been suggested that a chemotactic agent is responsible for the migration of mononuclear cells (see reviews by Spector & Willoughby, 1963 and Curran, 1967). However, an agent which has a chemotactic effect on mononuclear cells has yet to be identified. Alternatively it has been suggested that mononuclear cells migrate to the site of tissue injury as a result of random motion. Ham & Hurley (1965) showed that the escape of carbon particles from small venules at the site of inflammation, induced by intrapleural injection of turpentine into rats, only occurred when PMN migration and an increase in vascular permeability were coincident. This observation raises the possibility that the migration of mononuclear cells from small venules may be influenced by both PMN migration and increased vascular permeability. In this work it was shown that PMN can migrate under the influence of a chemotactic agent activated as a result of an interaction between tissue antigens and a complement fixing anti-tissue autoantibody. It seems significant, therefore, that other activated components of

complement have been shown to increase vascular permeability. For example, C'1 esterase has been shown to act on the C'3 and C'5 components of complement to produce a low molecular weight basic protein (anaphylatoxin) which is able to release histamine from mast cells (Müller-Eberhard, 1968).

It has been pointed out that the observable responses to tissue damage and to the introduction of foreign material in an immunized animal are very similar (Boyden, 1963). In both situations there occurs an initial increase in capillary permeability commencing a few seconds after the stimulus is given. The next observable event is the adherence of leucocytes to the venule walls which is followed by their emigration into the extravascular fluid (Florey, 1962; Wilson & Miles, 1965). Monocyte emigration commences at the same time as polymorph emigration but at a slower rate (Paz & Spector, 1962; Curran, 1967). Thus it can be seen that the inflammatory response is composed of a series of events rather than a single event. It is thought that different types of injury lead to variations in the relative intensity and duration of particular aspects of the response although the response follows a course which is essentially uniform (Spector & Willoughby, 1963). Boyden (1962,1963) showed that mixtures of normal adult rabbit serum and a variety of foreign material e.g. tuberculoprotein, macromolecules from plants, were chemotactic for PMN and evidence was presented that this response was a result of the interaction between the foreign material and "naturally occurring"

antibody in the rabbit serum. These observations led Boyden (1963, 1964) to postulate that the similarities in the local response to tissue damage and to foreign material may be a consequence of both responses being a result of antigen-antibody reaction. He argued that since tissue damage occurs frequently in all normal individuals then it would be expected that the sera of normal individuals would contain anti-tissue autoantibodies. The finding of anti-tissue autoantibodies in normal rat sera thus provides experimental evidence in support of Boyden's hypothesis. Moreover, it may be that the combination of these antibodies with the corresponding tissue antigen is the initial step in the clearance of tissue breakdown products. It has been shown that tissue antigen-anti-tissue autoantibody interaction activates the components of complement including the principal chemotactic agent responsible for PMN migration and there are a number of possible mechanisms whereby the attraction of PMN to the site of tissue injury may facilitate clearance of tissue breakdown products by other phagocytes. It is possible that the anti-tissue autoantibody acts as an opsonin for the phagocytosis of tissue breakdown products by macrophages. Finally, it may be that other components of complement, activated by the reaction between tissue antigens and anti-tissue autoantibodies play some part in the clearance of tissue breakdown products.

Autoimmunity and Tolerance

Possible Mechanisms of Evasion by Tissue Antigens

The work described in this thesis has shown that the sera of most normal rats contain autoantibodies reactive with tissue antigens. It appears, therefore, that rats are not immunologically tolerant to these antigens. Burnet (1959, 1963) has proposed two mechanisms by which an animal may be able to react immunologically against such self antigens. He suggested that an animal may not become tolerant to an antigen which is "sequestered" from the antibody forming cells during neonatal life. Alternatively he postulated that antigenic changes may take place in "self" components thus rendering them foreign to the antibody producing cells. However, in this work it was found that complement fixing anti-tissue autoantibodies could be detected in the sera of neonatal rats. In addition Weir & Pinckard (1967) have demonstrated that rats subjected to an intensive injection schedule of rat liver antigens from birth were quite able to respond to subsequent challenge with liver antigen or carbon tetrachloride. Clearly then the hypothesis that the anti-tissue autoantibody-forming cells are not effectively eliminated because the antigen is "hidden" from them during neonatal life is untenable. Equally, these observations render it unlikely that any changes which may occur in the antigenic constitution of these tissue antigens during ontogenesis, could account for the ability of rats to produce the

corresponding autoantibody.

The problem remains as to how it is that rats are able to produce autoantibodies reactive with tissue constituents. In the normal individual it is presumed that controlling mechanisms exist which prevent the antibody-forming tissues from reacting against host antigens. Thus it may be that the ability of rats to produce autoantibody against tissue antigens represents a failure of this "tolerance maintaining" mechanism i.e. tolerance to these tissue antigens is broken. On the other hand it may be that these tissue antigens evade or circumvent the normal tolerance inducing mechanism.

The results described here (Section 1 & 8) showed that rats were able to produce autoantibodies in response to stimulation from the corresponding tissue antigens from early in life. Thus it appears that tolerance to these tissue antigens is never induced. There is, furthermore, an impressive body of evidence which suggests that once tolerance to a particular antigen is firmly established it may never be abrogated at the cellular level. However, a number of claims that tolerance can be broken have appeared in the literature and it appears necessary to examine the evidence on which these claims are based.

Cross-reacting antigens and tolerance evasion

Weigle (1961) found that rabbits rendered tolerant to bovine serum albumin (BSA) could be stimulated to synthesise antibody

capable of binding BSA by immunisation with appropriate cross-reacting antigens. This phenomenon was originally interpreted as a demonstration of the breakage of tolerance. However, it was found that after such "anti-BSA" sera had been precipitated at equivalence with the cross-reacting antigen used for immunization, they bound essentially no I^{131} BSA in the presence of a large excess of the cross-reacting antigen, as measured by Farr's (1958) technique. Similarly, Paul, Siskind & Benacerraf (1967) found that rabbits rendered immunologically tolerant to BSA produced antibodies which precipitated BSA after challenge with dinitrophenyl (DNP)-BSA. Hapten inhibition and binding studies revealed that these "anti-BSA" antibodies were not directed against the DNP group. However, the "anti-BSA" antibodies were of extremely low affinity as compared with those produced by control animals immunized with DNP-BSA. These results were taken to show that those cells producing antibodies best adapted to the antigenic determinants of BSA remained inhibited in the tolerant animals and it was concluded that no true termination of tolerance at the cellular level had occurred. Rose & Cinader (1967) drew similar conclusions from their studies of the reactivity of rabbits rendered tolerant to human albumin (HA). Such rabbits were challenged with azo-derivatives of HA and it was found that they produced antibody which agglutinated HA coated sheep erythrocytes. Haemagglutination inhibition studies of these sera showed that the "anti-HA" antibodies were directed against conformationally

altered antigenic determinants or antigenic determinants containing the azo groups. Since this antibody was not directed against determinants to which tolerance had been induced Rose & Cinader considered that termination of tolerance had not taken place and suggested that this phenomenon would be better termed "circumvention" of tolerance (Cinader, Rose & Yoshimura, 1967).

It has been demonstrated that cells from tolerant animals remain unresponsive when tranferred to X-irradiated syngeneic recipients. For example, Dietrich & Weigle showed that spleen cells from mice rendered tolerant to human γ-globulin (HGG) did not confer the ability to respond to challenge injections of HGG when transferred to X-irradiated syngeneic recipients. Mcgregor, McCullagh & Gowans (1967) transferred thoracic duct lymphocytes from rats "tolerant" to sheep erythrocytes to X-irradiated syngeneic recipients. They challenged the recipients with sheep erythrocytes and detected no haemolysin response. In contrast, if the "tolerant" lymphocytes were incubated <u>in vitro</u> at 37°C. for 8-12 hours in serum-free medium prior to transfer then a haemolysin response could be detected in the recipients. This, it was claimed represented a breakage of tolerance. However, other workers have shown that it is difficult to render rats tolerant to sheep erythrocytes (Bauer, Peckham & Osler, 1956; Nossal, 1958). Moreover, Day & Farr (1966) have shown that the sera of rabbits receiving daily intravenous injections of BSA may contain anti-BSA antibodies which can only be detected after excess BSA has

been removed from the sera. These observations suggest that the lymphocyte population from rats "tolerant" to sheep erythrocytes may contain non-tolerant cells.

The observations described above indicate that tolerance at a cellular level cannot be terminated. However, they clearly demonstrate that in an animal tolerant to a particular antigen, antibodies which will react with the original tolerance inducing antigen can be evoked by immunization with an appropriate cross-reacting antigen. This raises the possibility that tolerance to certain tissue antigens might be evaded by immunization with cross-reacting antigens such as bacteria, which are known to share antigenic determinants with tissue antigens. It is known that evasion of tolerance can be prevented by injecting the original inducer together with the cross-reactive antigen. Thus Weigle (1964 b) and Humphrey (1964) immunized rabbits tolerant to bovine serum albumin (BSA) or human serum albumin (HSA) with suitable cross-reacting antigens and detected antibodies which bound the tolerance inducing antigen. In contrast they found that rabbits rendered tolerant to BSA or HSA and challenged simultaneously with the appropriate cross-reacting antigen together with the tolerance inducing antigen, failed to produce antibodies capable of binding the original tolerance inducing antigen. In this work it was found that young rats exposed to tissue antigens by CCl_4 induced liver damage had higher levels of complement fixing anti-tissue antibody than "normal" littermate controls (Section 8). Moreover,

Weir & Pinckard (1967) have injected tissue antigens into rats commencing shortly after birth. This procedure was found not to affect the ability of these rats to produce complement fixing anti-tissue antibody on subsequent challenge with tissue antigens. Consequently, it seems likely that tolerance to these tissue antigens is not being evaded by immunization with cross-reacting antigens, since rats cannot be made tolerant by exposure to these tissue antigens.

Other factors affecting induction of the tolerant state:

Effect of Antibody

A study of the literature revealed a number of factors which might affect the induction of tolerance to a particular antigen (see Introduction p. 6). For example, it has been demonstrated that the induction of tolerance by neonatal exposure to a particular antigen can be prevented by the simultaneous administration of the corresponding anti-serum (Friedman, 1965; Havas & Senff, 1967). In rats some maternal antibody is known to be transferred to the foetus via the yolk sac (Good & Papermaster, 1964). These findings suggested that if sufficient specific maternal antibody could be transferred to the foetus then it might interfere with the induction of tolerance to the corresponding antigen. However, in this work it was found that litters from mothers with high levels of circulating anti-bovine serum albumin (BSA) antibody, were as susceptible to the induction of tolerance to BSA as litters from

"normal" mothers (Section 8, Table 32). This maternal anti-BSA antibody is likely to be mainly of the IgG type (see Table 13) which is known to be selectively transferred to the foetus (Sterzl & Silverstain, 1967). It was found that the anti-tissue antibody is mainly of the IgM type. It therefore seems unlikely that sufficient maternal anti-tissue antibody could be transferred to the foetus to interfere with the induction of tolerance to the corresponding tissue antigens.

Effect of physical state of antigen

There is evidence that if an animal is exposed to an antigen then the physical state of the antigen may determine whether the animal is rendered tolerant or whether it gives an immune response (see Introduction p.10). For example, it has been demonstrated that mice and rabbits can be rendered tolerant to bovine γ-globulin and bovine serum albumin by the injection of aggregate free preparations of these antigens. In contrast mice and rabbits injected with aggregated preparations of these antigens exhibit an immune response (Dresser, 1962; Dietrich & Weigle, 1964; Thorbecke & Benacerraf, 1967; Pinckard, Weir & McBride, 1967). Similarly, Ada, Nossal & Austin (1965) showed that tolerance to the soluble flagellin antigen of Salmonella adelaide could readily be induced by injection of flagellin into neonatal rats. However, they failed to induce tolerance to the particulate flagella antigen. Rats injected with flagella twice weekly from birth were able to

respond to subsequent challenge with flagella.

It has been shown that the liver antigen which fixes complement with the anti-tissue antibody induced by toxic liver damage is localised in the sedimentable fractions of rat tissue homogenates (Weir, 1963; Elson, 1965; Pinckard & Weir, 1966; Section 4. Fig. 10). Similarly it was found that the complement fixing anti-tissue activity in normal rat sera was directed against sedimentable components of tissue homogenates (Fig. 8 & 9). These observations led to the suggestion that the failure of rats to become tolerant to these tissue antigens is associated with the physical state of these antigens. It was postulated that following cell death both soluble and particulate tissue antigens would be released (Weir, 1967). A rat might then become tolerant to the soluble antigens but exhibit an IgM immune response towards the particulate antigens (Elson, 1965; Weir, 1967; Pinckard & Weir, 1966; Weir & Pinckard, 1967).

In this work two experiments were carried out to test this hypothesis. In the first, the ability of rats to respond to an aggregate free preparation of tissue antigens was tested. Rats were injected with a preparation of soluble liver antigens (F_7) and their sera taken at intervals and tested in the complement fixation test against F_7. The results (Section 8) showed that rats responded to the injection of F_7 by the production of complement fixing IgM autoantibodies. However, it was found that these autoantibodies also reacted with antigens from particulate fractions

(Table 30) and could be absorbed out with them (Table 31). This indicates that the antigens in F_7, against which rats responded, were fragments of particulate tissues broken down by the in vitro homogenisation procedure used to prepare the tissue antigens. On injection into rats such antigens would almost certainly be aggregated by the anti-tissue antibodies already present.

The second experiment was designed to determine whether or not an aggregated preparation of a soluble antigen was more or less effective than the same soluble antigen in its ability to induce tolerance in neonatal rats. Rats were given a single injection within 24 hours of birth of either alum precipitated bovine serum albumin (BSA), soluble BSA or aggregate free BSA. The results (Table 24) showed that tolerance was more effectively induced with alum precipitated BSA than either soluble BSA or aggregate free BSA. The obvious explanation for this apparently discrepant result is that BSA was slowly released from the alum thus maintaining a constant concentration of BSA in contact with the lymphocytes over a period of time. It is difficult on this theory to explain why, on a weight basis, alum precipitated BSA is about 10 times more effective in inducing tolerance in neonatal rats than aggregate free BSA. It was found that 0.5 mg. alum precipitated BSA induced hyporeactivity in 10/14 rats (Table 32) whereas 5 mg. centrifuged (aggregate free) BSA induced hyporeactivity in 3/6 (Table 24).

A purely speculative explanation for these results is offered.

Alum precipitated BSA is phagocytosed but this BSA is unable to initiate the production of antibody either because the neonatal macrophages are unable to "process" it and/or because the corresponding lymphocytes are unable to respond to the stimulus. On the other hand macrophages are able to release this BSA in a tolerogenic form, and high concentrations of tolerogenic BSA may thus be built up in the vicinity of the lymphocytes. It has been shown that the phagocytes of neonatal rats are able to ingest such foreign material as carbon particles and P^{32} Salmonella typhimurium (Reade & Jenkin, 1964). However, neonatal rat phagocytes were found not to kill ingested Salmonella typhimurium although the phagocytes of adult rats rapidly killed ingested Salmonella typhimurium. These observations suggest that a comparison of the exocytic and degradative capacities of macrophages from neonatal and adult animals may shed some light on the mechanism of tolerance induction.

The original theory as to how particulate antigens and particulate tissue antigens in particular evade the tolerance inducing mechanism appears to require some modification in the light of the findings described above. Whether or not an animal becomes tolerant to a given antigen appears to depend on a balance between factors which favour immunisation and factors which favour the induction of tolerance (see review by Dresser & Mitchison, 1968). Similarly, in the case of particulate tissue antigens it may be that a number of factors combine to induce a state of immunity rather than tolerance. Thus, although it may be that particulate tissue

antigens are phagocytosed and thereby prevented from coming into direct contact with the corresponding lymphocytes, it seems unlikely that this is the sole factor involved in their evasion of the normal tolerance inducing mechanism. Paralysis of the type associated with pneumococcal polysaccharides or synthetic D amino acids (see Introduction p. 12) would seem unlikely to occur as it would be expected that macrophages possess enzymes capable of degrading tissue antigens. This suggestion is substantiated by the work of Weir & Pinckard (1967) who injected rats within 24 hours of birth with 200 mg. of liver antigen and followed this with injections of 100 mg. every two days for five weeks. This treatment was found to have no effect on the ability of these rats to produce complement fixing anti-tissue autoantibodies in response to challenge of liver antigen. This observation also suggests that even if particulate tissue antigens do come into direct contact with the corresponding lymphocytes they are unable to induce tolerance. A possible reason for this may be that, due to the size of the tissue antigens an effective interaction between the "recognition site" on the lymphocyte and the corresponding antigenic determinant group cannot take place.

Effect of Maturity of Antibody Forming Cells

A further possibility which requires some consideration is that the anti-tissue autoantibody forming cells mature early in ontogenesis and that rats are thus able to respond to antigenic

stimulation by the corresponding tissue antigen from early in development. It seems significant that the complement fixing anti-tissue antibodies were shown to be IgM in type and there is evidence that animals are able to produce IgM antibodies, in response to stimulation by certain antigens, very early in development. For example human infants injected with the flagella antigen of Salmonella paratyphi B have been found to produce IgM agglutinins 7 days later (Smith & Eitzman, 1964). The 7S response was delayed and IgG agglutinins were not detected until the infants were 30-40 days old. In contrast IgG agglutinins were detected in the sera of adults about 8 days after immunisation. Similar results have been obtained using the same flagella antigen in rabbits (Bellanti, Eitzman, Robbins & Smith, 1964) and phageϕX 174 in premature human infants (Uhr, Dancis & Franklin, 1962). These results suggest strongly that the IgM antibody forming cells reach maturity and are able to respond to antigenic stimulation early in ontogenesis. In this work it was found that complement fixing anti-tissue antibodies could be detected within 24 hours of birth. Furthermore, neonatal rats injected with CCl_4 were found to have higher titres of complement fixing anti-tissue antibody than untreated littermate controls. This indicates that rats are able to respond to stimulation by tissue antigens early in life.

The facility with which tolerance to an antigen can be induced appears to be inversely related to the ability of that antigen to elicit an immune response (see review by Dresser & Mitchison, 1968).

If the IgM antibody forming cells are the first to mature in ontogensis, then in neonatal animals the ease with which an antigen induces tolerance will be inversely related to its ability to elicit an IgM response. The anti-tissue antibody described in this work was found to be an IgM antibody. Thus, it may be that the ability of the corresponding tissue antigens to evoke an IgM response is an important factor in their ability to evade the tolerance inducing mechanism. Moreover the possibility arises that this is a general phenomenon applying to other antigens. There is evidence that those particulate antigens which are poor tolerogens (see Introduction p. 10), stimulate high IgM responses. For example Ada, Nossal & Austin (1965) found that rats injected with polymerized flagellin produced high titres of IgM antibody in the week following immunization as measured by the bacterial immobilisation technique. In contrast, little or no IgM antibody was detected in the sera of rats injected with the soluble flagellin antigen although the peak titres of IgG antibody were similar to those obtained in rats injected with an equal weight of polymerised flagellin.

The results discussed above suggest that a number of factors may be involved in the evasion of the tolerance inducing mechanism by the tissue antigens which react with the anti-tissue antibody found in "normal" rat sera. These factors appear to be associated with the capacity of the tissue antigens to elicit an immune response rather than tolerance in neonatal rats. It is suggested

that the particulate nature of the tissue antigens combined with the ability of the macrophages to degrade and "process" these antigens and the early maturity of the corresponding antibody forming cells are of prime importance in the attainment of this state of immunity. It remains to discuss the significance of the ability of animals to respond immunologically to such tissue antigens.

Components of the Immune Reaction to Tissue Antigens

There is evidence that some types of autoantibody potentiate tissue damage whilst others appear to have an inhibitory effect (see Introduction p.13). Thus Brown, Glynn & Holborow (1967) have demonstrated that the presence of anti-testicular autoantibodies is an essential factor in the development of testicular lesions in allergic experimental orchitis in guinea pigs. Appel & Bornstein (1964) have described an IgG $\gamma 2$ demyelinating and glial-toxic antibody in the serum of rats and rabbits with experimental allergic encephalomyelitis (EAE). On the other hand Paterson & Harwin (1963) have shown that a complement fixing IgM anti-brain antibody in the serum of Wistar rats has a suppressive effect on the induction and severity of EAE as high levels of complement fixing IgM anti-tissue antibody were found in the sera of Wistar rats which were shown to be resistant to the induction of EAE. In contrast no complement fixing IgM anti-tissue antibody was detected in Lewis rats which were shown to be highly susceptible

to EAE. Moreover, the injection on alternate days of complement fixing IgM anti-brain antibody from Wistar donors, was found to suppress the symptoms of EAE in appropriately sensitised Lewis recipients. Finally, the occurrence of brain lesions and cytotoxic activity of the serum was found to be coincident in Lewis rats given lymph node cells from syngeneic donors with EAE.

Coombs (1958) suggested that the autoantibodies found in autoimmune disease states may be "exaggerated" or "enhanced" levels of those having some physiological function in normal animals. In this work evidence was presented (see Discussion p.135) that the IgM anti-tissue autoantibody, found in "normal" rat sera has a physiological role; perhaps initiating and facilitating the clearance of tissue breakdown products. In addition it was found that tissue damage stimulated the production of this autoantibody. It seems significant therefore that most of the autoantibodies found in "normal" animals and the pathogenic autoantibodies found in autoimmune diseases appear to be directed against damaged tissue (see Introduction p.13). For example the cytotoxic antibodies found in patients with throiditis and ulcerative colitis act only on cells which have been trypsinised prior to testing (Pulvertaft et al, 1959; Irvine, 1962; Broberger & Perlman, 1963). Levine & Wenk (1967) transferred cells from rats with allergic encephalomyelitis to syngeneic donors with brain damage. The sensitised cells were found to localise in areas of the brain already damaged, and this was taken to indicate that the target antigen recognised

by the cells was damaged tissue. Autoantibodies characteristic of those found in autoimmune disease states have been detected in the sera of "normal" animals. Thus low levels of both "warm" and "cold" type anti-erythrocyte autoantibody, characteristic of the autoimmune haemolytic anaemias, have been found in "normal" individuals (Delage, 1958; Jancovii, 1954). Similarly the cytotoxic effect of "normal" autologous serum on trypsinised rat and rabbit epidermal cells and trypsinised human synovial cells has been attributed to the action of anti-tissue autoantibodies (Terasaki & Chamberlain, 1962; Clarris & Fraser, 1968). Furthermore, Spooner (1964, 1965) has demonstrated the presence of a cytolytic IgG anti-testicular autoantibody in the sera of normal guinea pigs, rats, rabbits, cats and dogs.

These observations suggest that in "normal" animals an equilibrium between the different facets of the immune response to tissue antigens is established and maintained. There is evidence that some components of the immune response to a particular antigen can be temporarily inhibited by prior contact with the antigen. Thus it has been found that the usual IgG $\gamma 2$ and delayed type hypersensitivity response to the injection of antigens in Freund's complete adjuvant in guinea pigs is inhibited by prior immunization with the antigen on alum or in incomplete adjuvant (Asherson & Stone, 1965; Loewi, Holborow & Temple, 1966). The temporary state of this inhibition was shown by the finding that guinea pigs were able to exhibit a full delayed type hypersensitivity response

about 1½ months after the "deviating" injection (Loewi, Holborow & Temple, 1966). The work described in this thesis has shown that the sera of "normal" rats contains an IgM anti-tissue autoantibody which appears to be produced in response to antigenic stimulation by tissue breakdown products. Whether or not an IgG anti-tissue autoantibody is produced in response to the same stimulus was not unequivocally demonstrated. However, isolated reports have appeared of the detection of IgG anti-tissue autoantibodies in rabbits following the injection of heterologous tissue antigens (Ashenson & Dumonde, 1962, 1963) or after cryosurgical tissue damage (Shulman et al, 1968). It seems unlikely that this breakthrough to IgG anti-tissue antibody production represents a termination of a tolerant state (see Discussion p.137). On the other hand the finding that it is difficult to elicit an IgG response to tissue antigens in rats and rabbits suggests that this component of the immune response to tissue antigens is inhibited. It is, furthermore, well established that animals whose sera contain IgM anti-tissue antibodies of the type described here, are resistant to the induction of experimental allergic diseases (Paterson, 1966; Weir & Pinckard, 1967). In contrast animals such as Lewis rats and guinea pigs, in whose sera IgM anti-tissue antibodies have not been detected (Paterson, 1966, Section 1) are relatively susceptible to the induction of experimental allergic diseases (see for example Stone, 1961; Lee et al, 1965). These observations suggest that the IgM anti-tissue anti-

body itself inhibits other facets of the immune response to tissue antigens and/or that its presence is a reflection of this inhibition. In this respect it may be significant that passively administered homologous anti-sheep erythrocyte antibodies have been found to exert a suppressive effect on the haemolysin response to sheep erythrocytes in rats and rabbits (Rowley & Fitch, 1964; Pearlman, 1966). However, it has been shown that the development of delayed hypersensitivity and the IgG $\gamma 2$ response to the injection of an antigen in a suitable form is paralleled by the occurrence of a granulomatous lesion at the site of injection (White, Coons & Connelly, 1955; Wilkinson & White, 1966). Such lesions are characterised by relatively weak polymorphonuclear leucocyte (PMN) infiltration and mitosis of mononuclear cells at the site of chronic inflammation (Spector, 1967). These observations suggest that the cellular events which follow the injection of an antigen may affect the balance between the different components of the immune response to the antigen. In this work evidence was presented which indicated that, in rats, the interaction of tissue antigens and IgM anti-tissue autoantibodies liberated a chemotactic agent which effected the migration of PMN to the site of inflammation (see Discussion p. 126). This observation raises the possibility that the IgM anti-tissue autoantibody influences the cellular events following tissue damage with the result that the IgM response to tissue damage is maintained whereas other facets of the response to tissue antigens remain inhibited.

If there is an equilibrium between the different aspects of the immune response to tissue antigens then it would be expected that an upset of this equilibrium could result in a disease state. It has been found that the development of delayed type hypersensitivity and the IgG γ2 response to an antigen in guinea pigs, is considerably enhanced by the injection of the antigen in the form of an emulsion in Freund's complete adjuvant (White, Coons & Connelly, 1955; Wilkinson & White, 1966). Similarly, the injection of tissue antigens in the form of an emulsion in Freund's complete adjuvant, has been found to facilitate the induction of experimental allergic diseases (see Introduction p.18). Moreover, as has been discussed above, both circulating anti-testicular autoantibodies and delayed hypersensitivity to testicular antigens are required for the development of allergic testicular lesions in the guinea pig (Brown, Glynn & Holborow, 1967). Since IgG γ I anti-brain antibodies have been found to play no part in the development of experimental allergic encephalomyelitis in guinea pigs (Falk, Heinze, Kies & Alvord, 1968) then it seems likely that the autoantibodies essential for the development of allergic lesions in guinea pigs are IgG γ2. These observations thus suggest that exaggerations in the delayed type hypersensitivity and IgG γ2 response to tissue antigens may be responsible for the development of allergic lesions. In support of this contention is the finding that both the delayed type hypersensitivity and the IgG γ2 response to an antigen injected in Freund's complete adjuvant,

and the induction of allergic lesions following the injection of tissue antigens in Freund's complete adjuvant, are inhibited by prior injection of the antigen alone, in incomplete adjuvant or on alum (Asherson & Stone, 1965; Loewi, Holborow & Temple, 1966; Brown, Glynn & Holborow, 1963; Weigle et al, 1967). Further evidence in support of this hypothesis comes from the known role of delayed hypersensitivity to tissue antigens in auto-immune diseases (see Introduction p.19). Moreover, it seems significant that a number of autoantibodies which appear to potentiate the production of allergic lesions are of the IgG γ2 type e.g. the cytotoxic antibody in patients with thyroiditis (Irvine, 1962) and the demylinating antibody in patients with active multiple sclerosis (Bornstein & Appel, 1965). Finally, there is evidence that selective depression of IgG γ2 and delayed type hypersensitivity is associated with the failure to reject tumours (Mildenhall & Nelson, 1968; Augustin, personal communication). This observation raises the possibility that pathological states other than auto-immune disease may be potentiated by the upset of the equilibrium between the components of the immune response to tissue antigens.

Ehrlich and Morgenroth (Ehrlich, collected papers, 1957) in their third communication on haemolysins reported their failure to elicit autolysins in goats. They took these experiments "to show how very complicated the conditions are when the material of its own body is absorbed by an organism". In addition they con-

cluded that such an absorption will not lead to the permanent injury of an organism unless certain "regulating contrivances" fail. The work described here suggests that Ehrlich's "regulating contrivances" are mediated through a state of autoimmunity against tissue breakdown products and that Ehrlich's "horror autotoxicus" is a consequence of the disturbance of this physiological state of autoimmunity.

SUMMARY

The work reported in this thesis has shown that the sera of most "normal" rats contains an anti-tissue autoantibody. "Normal" rat sera were found to be active against various rat tissue and organ homogenates in the complement fixation and passive haemagglutination tests. The activity was eluted with the IgM and α_2 macroglobulins from Sephadex G 200 and was associated with the high molecular weight serum components sedimenting with the IgM on sone ultracentrifugation in sucrose density gradients. The tissue antigens which are active in the complement fixation test against normal rat sera were found to be associated primarily with "mitochondrial" fractions of tissue homogenates. Mitochondrial preparations of rat liver (F_3) were found to take up IgM and complement components selectively from "normal" rat sera. Rabbits and guinea pigs, which were immunized with F_3 exposed to various rat serum components, produced antibodies against rat IgM and complement components. The uptake of rat IgG by F_3 was not unequivocally demonstrated.

The complement fixing ability of the anti-tissue autoantibody present in "normal" rat sera was reduced or removed by heating the sera at 56°C. for 30 minutes. The response to tissue damage was shown to be an increase in the production of predominantly heat stable (56°C. for 30 minutes) anti-tissue antibody. The primary binding of rat anti-bovine serum albumin (BSA) sera (or IgM fractions) was found to be unaffected by heating at 56°C. for 30 minutes although the complement fixing activity of these sera was

reduced or removed. These findings were interpreted as showing that heating at 56°C. affects the Fc portion of the immunoglobulin molecule rather than its antigen binding Fab fragment. Similarly, the primary binding of the anti-tissue autoantibody was found not to be destroyed by heating and it was considered that changes in the heat lability of the anti-tissue autoantibody were a reflection of changes in the Fc portion of the molecule.

Studies directed towards defining a possible physiological role of the anti-tissue autoantibody described above suggested that it may be implicated in the removal of tissue breakdown products. Mixing normal rat sera with particulate sub-cellular components of rat liver was found to induce migration of polymorphonuclear leucocytes (PMN). Evidence was presented which indicated that this attraction of PMN was mediated by a chemotactic agent activated as a result of the interaction of tissue antigens and the complement fixing anti-tissue autoantibody.

It was found that the sera of neonatal rats contained complement fixing autoantibodies and this was taken to indicate that tolerance to the corresponding tissue antigens was never induced. An enquiry as to how these tissue antigens evade the normal tolerance inducing mechanism was made. It was found that:

1. Litters from rats with high levels of circulating anti-BSA antibody were as susceptible to the induction of tolerance to BSA as litters from "normal" rats.
2. Tolerance was more effectively induced to BSA in neonatal

rats with alum precipitated BSA than with BSA alone or aggregate free BSA.

3. The injection of a soluble preparation of tissue antigens into rats stimulated the production of anti-tissue auto-antibody. This antibody was directed against antigenic determinants shared with sedimentable tissue components.
4. Higher levels of anti-liver antibody were found in neonatal rats given CCl_4 than in untreated littermate controls.

It was suggested that the particulate nature of the tissue antigens together with the ability of macrophages to degrade and "process" these antigens and the early maturity of the corresponding antibody forming cells may be of prime importance in their evasion of the normal tolerance inducing mechanism.

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